Restriction Fragment Polymorphisms in Candidate Genes Associated with Hypertension

Thesis

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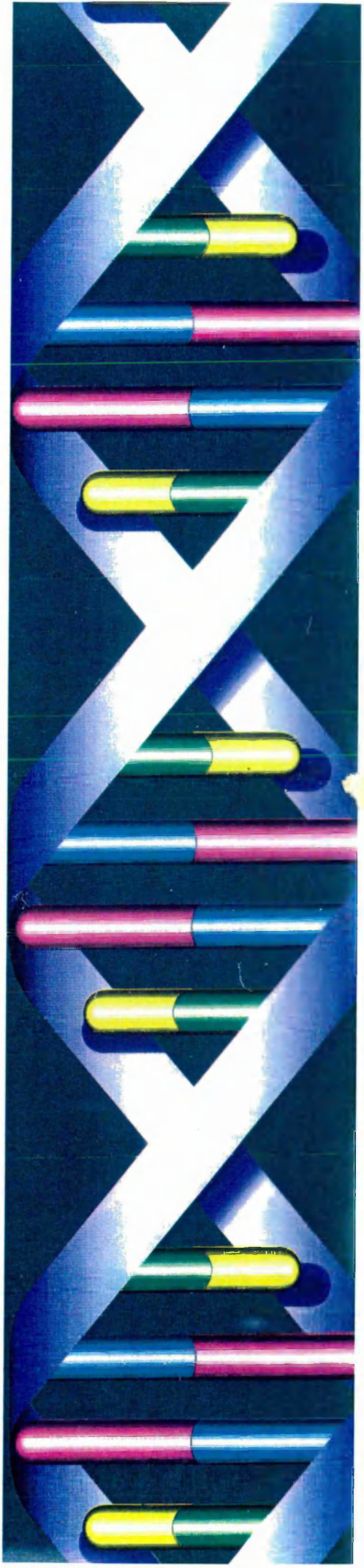
RESTRICTION FRAGMENT POLYMORPHISMS IN CANDIDATE GENES ASSOCIATED WITH HYPERTENSION.



by

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Restriction fragment length polymorphisms in candidate genes associated with hypertension.

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Abstract

This thesis describes an investigation of the role of genetic variation and its association with hypertension.

Using molecular biology techniques it is possible to analyse DNA from subjects and distinguish variants of genes that might be involved in blood pressure regulation as potential predictors of essential hypertension.

Restriction fragment length polymorphisms (RFLPs) at the gene loci for renin, atrial natriuretic peptide, glucocorticoid receptor and kallikrein have been identified and their frequency determined in three different study groups to establish an association with blood pressure.

Measurement of renin, atrial natriuretic peptide and other physiological parameters associated with blood pressure was carried out on selected individuals, and the results correlated with RFLPs.

The main conclusions from the study were:-

1. There was no significant association of RFLPs with blood pressure.
2. There was a striking and significant ethnic difference for several of the RFLPs studied.
3. There was no association of physiological measurements with any of the RFLPs.

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CHAPTER 1. INTRODUCTION.

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SECTION 1. Genetic disease and DNA technology.

Over the last fifteen years the techniques in molecular biology have advanced rapidly, resulting in major breakthroughs in the study of Human genetic diseases.

These can be divided into several categories.

Some have a defect in a single known gene, and both the gene product and the gene can be studied, of which the best example is sickle-cell anaemia. A single base change in the DNA sequence that codes for the ß - globin protein, results in a change in the amino acid translation, and valine is substituted for glutamic acid. (Ingram. 1957)[1]. This changes the charge on the protein and therefore causes a variation in migration rate, as resolved by electrophoresis.

Many hundreds of conditions fall into this group, where biochemical tests are used to determine the disease and over the last five years, with the use of gene probes, the defective genes have been identified.

For other diseases where the pattern of inheritance indicates a single gene defect, for example, Huntington's chorea and Adult polycystic kidney disease, there are no detectable biochemical tests, and gene probes have been used as linked diagnostic markers where the gene defect is unknown.

(Gusella. 1983)[2]. (Reeders. 1985)[3].

In some cases the gene itself has been identified, by the method of 'reverse genetics', and the defect at protein level

determined. One of the major diseases in this group is Duchenne Muscular dystrophy.(Hoffman. 1987)[4]

In the case of genetic diseases where one or several unknown genes interact to cause the development of the pathology it is possible to use similar DNA techniques to study 'candidate genes'.

The advantage of using DNA is that, with only a few exceptions, all cells contain the same genome and tissue that is easily obtainable such as blood leukocytes, can be used to analyse genes that are expressed in other, harder to obtain tissues.

This study investigates the genetic variation of 'candidate genes' and their contribution to the development of high blood pressure. The genes of interest were chosen as those that are involved in maintaining blood pressure homeostasis and had been cloned and available as probes. The difficulty in looking at variations in the genes is to assess whether these changes contribute to the development of the disease, high blood pressure, or whether, through the slow changes of evolution, these are neutral changes that have no pathological significance.

These variations can be identified using Restriction fragment length polymorphisms.(RFLPs)

Section 2. Restriction fragment length polymorphisms.(RFLPs)

In the last twenty years the use of bacterial restriction endonucleases has been a major factor in the advancement of DNA technology. (Review by Malcolm. 1981)[5].

These enzymes are part of the bacterial defence mechanism against invasion by foreign DNA, such as viruses. They function by recognising a unique DNA sequence and cutting at a specific site either in the recognition sequence or adjacent to it. A feature of most of these recognition sequences is that they are palindromic, ie. that the sequence in one strand is repeated in reverse in the second strand. The restriction endonuclease EcoR I recognises the first strand sequence GAATTC and in the second reverse strand CTTAAG.

These enzymes are used to cut genomic DNA into fragments and, depending on how common these recognition sequences are , will result in a specific number and size of fragments. The human diploid genome consists of approximately 6 x 10^9 base pairs and may be cut into about one million discrete fragments between 100 to 100,000 base pairs in length. Only one fragment will contain the gene of interest, or two or three fragments if the enzyme used cuts within the gene. These fragments are detected by Southern blotting analysis. (Southern. 1975)[6].

Because of the specificity of the restriction enzyme sites, variations in the DNA sequences from one individual to another can result in differing numbers of sites and

therefore different sizes of DNA fragments after enzyme digestion. These size differences are termed Restriction Fragment Length Polymorphims, (RFLPs).

In the case of sickle-cell anaemia, the DNA mutation results in a substitution of amino acids but it also destroys the recognition sequence and cutting site for the enzyme Mst II. (Orkin. 1984)[7]. DNA digested with the enzyme Mst II and hybridised with a ß-globin gene probe will show restriction fragments of 1.1 and 0.2 Kb for the normal allele and only a 1.3 Kb fragment for the mutated or sickle-cell allele. The use of mutation specific restriction enzymes has a limited use due to the high specificity of the recognition sites.

However, RFLPs can be used as gene markers. If two genes are located close to each other on the same chromosome the likelihood of them being inherited together is higher than chance, and if the marker is close to the gene that causes the disease, it will cosegregate with the disease allele. If crossing over of the chromosomes ,in meiosis, occurs between the loci of the marker gene and the disease gene they will then not be inherited together, and only genes that have less than 50% recombination rate can be used in linkage studies. The more polymorphic markers close to or inside the disease gene region the higher the probability of finding genetic linkage.

An example, using an RFLP as a marker to a disease gene, is the polymorphism detected when using the restriction enzyme

Pvu II and the low density lipoprotein(LDL) receptor gene probe in a family with Familial Hypercholesterolaemia , FH . (Horsthemke. 1985)[8], where one of the allelic polymorphisms co-segregates with the disease. However this defect in the LDL- receptor gene is not common throughout the population and therefore can only be used as a predictor of FH in particular family studies.

Similarly, markers can also be used when looking for an association with a disease in the population. An example of a polymorphic protein marker is shown in the disease ankylosing spondylitis which is strongly associated with the histocompatibility antigen, HLA-B27. (Bodmer. 1978)[9]. This polymorphism only occurs in 5-10% of the general population, but 90% of patients who have ankylosing spondylitis have this HLA type.

Restriction fragment length polymorphisms have also been used as markers associated with a disease. The RFLP obtained using the restriction enzyme Hpa I and a ß-globin gene probe and the association with sickle cell anaemia, is one of the earlier examples. (Kan and Dozy. 1978)[10]. In certain populations, over 80% of individuals having a 13 Kb restriction fragment have the sickle-cell gene as compared to normal individuals who have a restriction fragment of 7.6 Kb. This association between the disease gene and a restriction fragment marker is termed 'linkage disequilibrium'.

Section 3. Southern blotting analysis.

The use of recombinant DNA technology now allows the direct detection of specific genes in Human DNA, by Southern blotting analysis. Many genes have now been cloned and are available as recombinant molecules containing cDNA copies of the mRNA, and are used as gene probes.

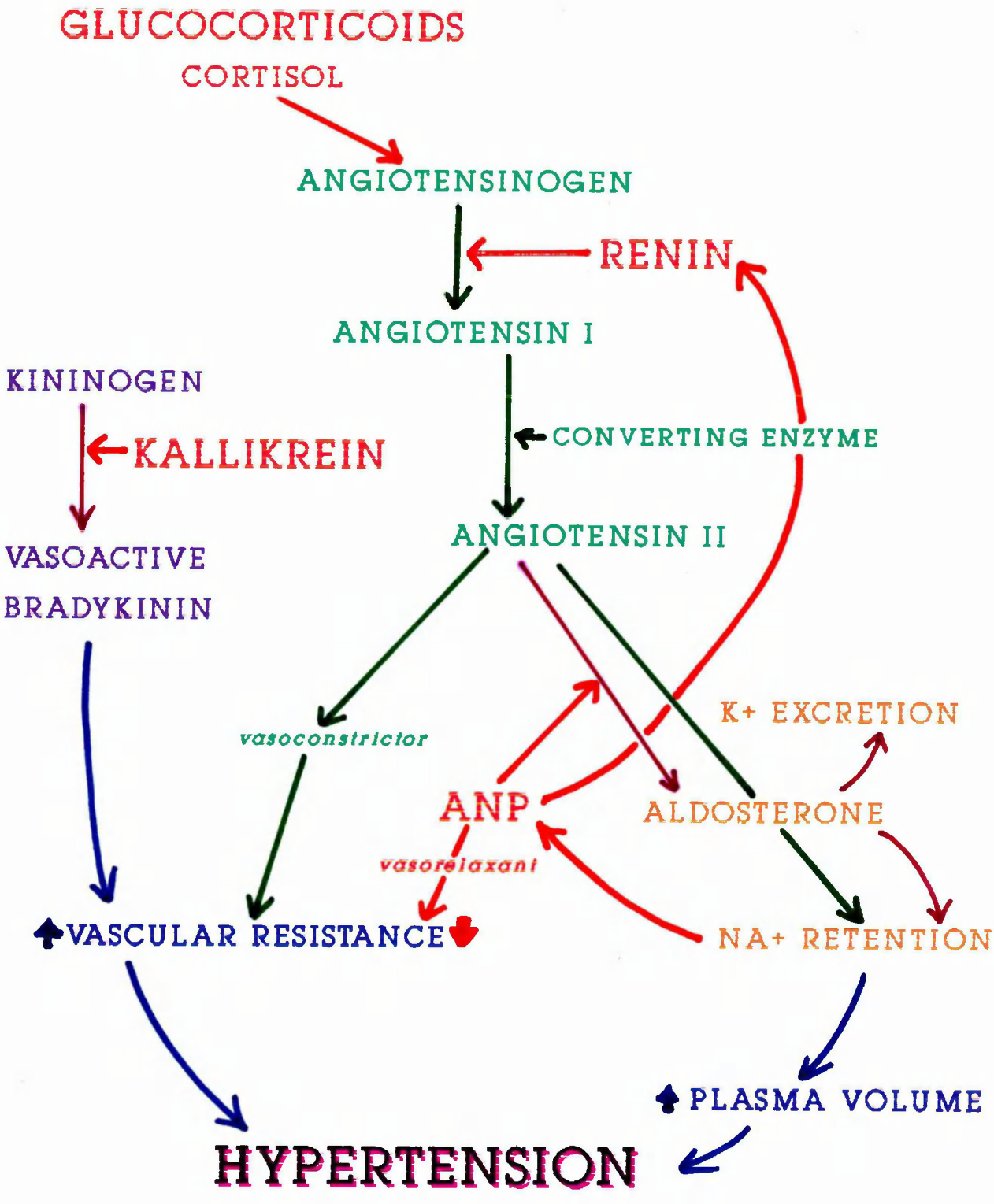
DNA is extracted from whole blood, as described in the methods section, and digested with a restriction endonuclease, under set conditions. The double stranded fragments obtained are separated by size, using agarose gel electrophoresis. The double stranded DNA is denatured to single stranded DNA by soaking the gel in strong alkali, and neutralised before being placed on absorbent paper with wicks into a tank of concentrated salt solution. A nylon membrane is placed on top of the gel with dry absorbent paper and towels on top of this. (See figure 11.). The salt solution is drawn up through the gel and carries the single stranded DNA out of the gel and onto the membrane to produce a replica of the DNA from the agarose gel. The DNA is fixed onto the membrane by 'baking' at 80^{o}C. The gene probe is radioactively labelled and rendered single stranded by denaturing the hydrogen bonds between the bases by heating to 95^{o}C for two minutes and cooling immediately to 4^{o}C.

The single stranded probe then pairs with the complementary DNA sequence on the membrane filter in the process known as hybridisation and the radioactivity is localised to a particular size fragment, detected by autoradiography.

Section 4. Candidate genes.

Since nothing is known about the genetic loci controlling blood pressure in humans, the only 'long-shot' method is to look at candidate genes for variation of blood pressure and associated disorders that might be involved in the development of hypertension. These would be genes where the expression products are physiologically known to contribute to the control of blood pressure. These controlling mechanisms involve a complex interaction, where the major determinants are :- cardiac output, blood vessel tonicity, renal function, including the renin-angiotensin-aldosterone system and other factors involved in diuresis and natriuresis. (See Figure 1.) The many pathways involved in this regulation show numerous potential candidate genes and those chosen in this study had been cloned by others and the clones made available to us for research studies.

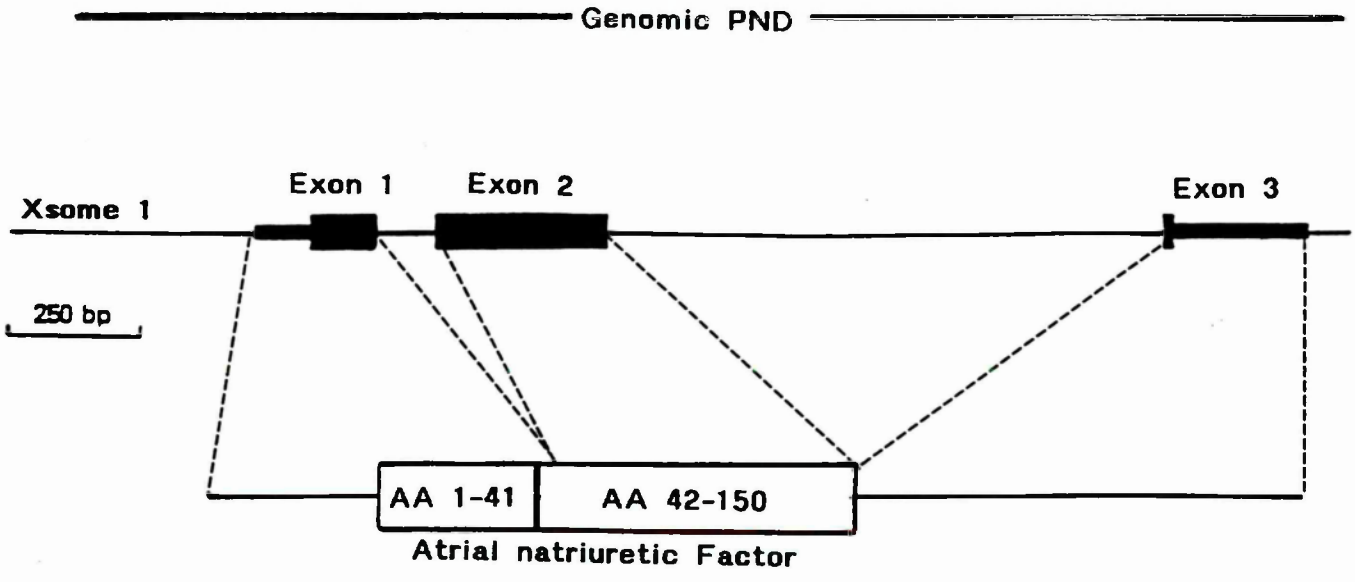
FIGURE 1. PHYSIOLOGICAL PATHWAYS INVOLVED IN BLOOD PRESSURE HOMEOSTASIS.



ATRIAL NATRIURETIC FACTOR AND PRONATRIODILATIN.

Atrial natriuretic factor, now more recently termed Atrial natriuretic peptide, is a circulating hormone produced in the cardiac atrial muscle cells from a larger precursor, pronatriodilatin, (PND). It is known to be important in the regulation of extracellular fluid volume by increasing the excretion of sodium from the kidney, most probably by increasing glomerular filtration rate. As well as having a potent natriuretic and diuretic action, it also acts as a relaxant on precontracted vascular smooth muscle, blocks angiotensin-induced aldosterone secretion by the adrenal cortex and inhibits renin secretion by the kidneys.
(Review by Needleman. 1985)[11].

SCHEMATIC REPRESENTATION OF THE ANF. AND PND. GENE.



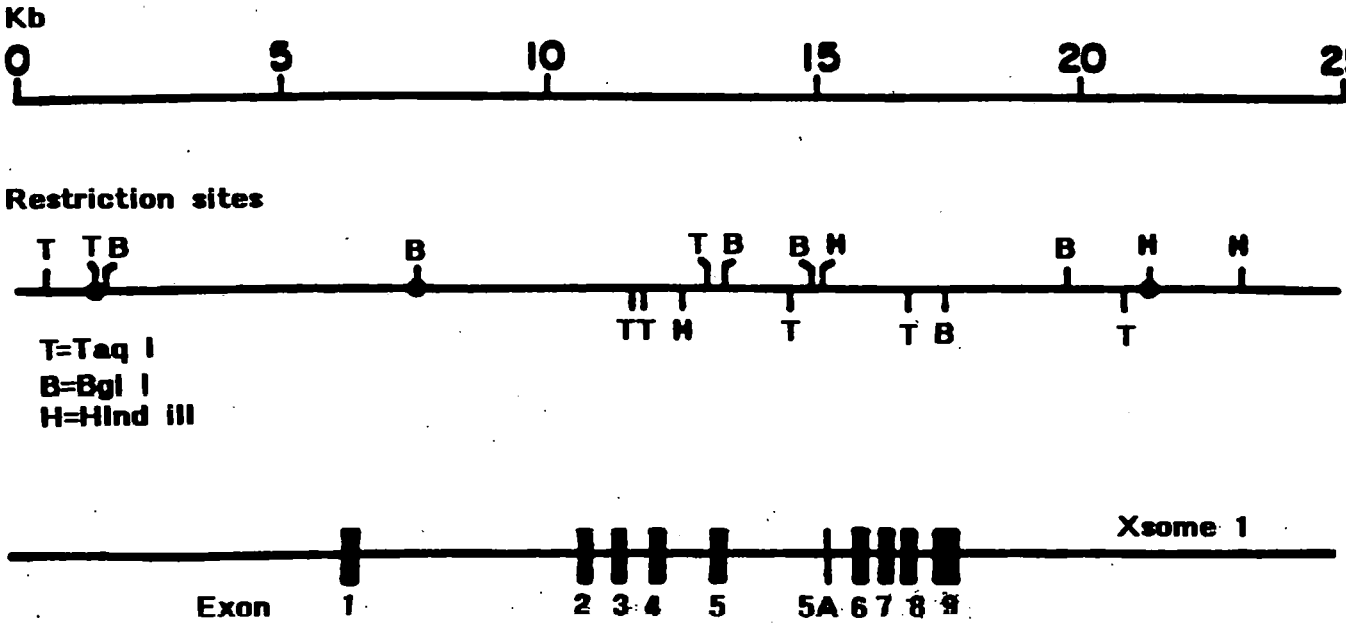
Reference. PND (Nemer. 1984)[12]

ANP (Zivin. 1984)[13]

RENIN.

Renin is an enzyme synthesized mainly in the juxtaglomerular cells of the afferent arteriole of the kidney, where it is stored as granules. It is released when the sodium ion concentration is low in the distal tubule region or when there is low blood flow to the kidneys. It cleaves the substrate angiotensinogen and forms angiotensin I which is then enzymatically converted to angiotensin II, a powerful vasoconstrictor which increases blood pressure and also brings about the release of aldosterone from the adrenal cortex, which, in turn, increases the reabsorption of sodium. (Review. Meade. 1983)[14]

SCHEMATIC REPRESENTATION OF THE RENIN GENE.

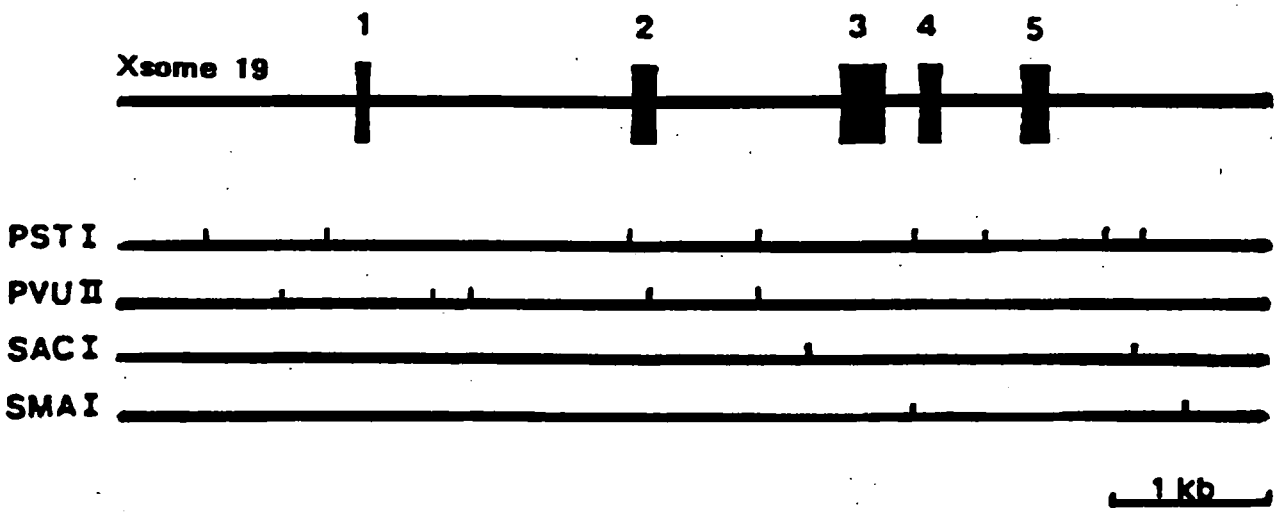


Reference. (Soubrier. 1983)[15]

KALLIKREIN.

Kallikrein is an enzyme derived from the precursor, prekallikrein , and acts on the substrate kininogen to form bradykinin or kallidin, which causes a fall in blood pressure. The mechanism by which this works is not entirely known but it is known to be a powerful vasodilator in conjunction with prostaglandins.(Review. Carretero. 1980)[16].

SCHEMATIC REPRESENTATION OF THE KALLIKREIN GENE.

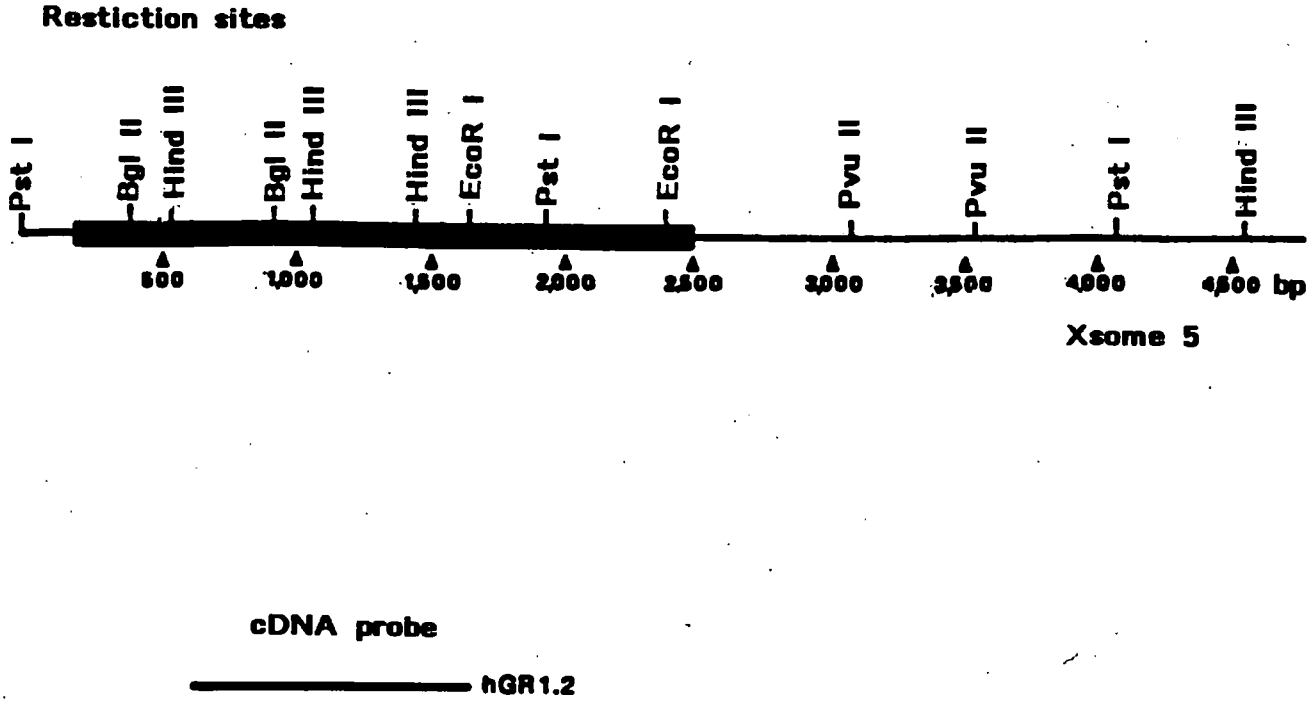


Reference. (Baker. 1985)[17]

GLUCOCORTICOIDS.

Glucocorticoids, or adrenal steroids, are not stored preformed and are synthesized and released by the action of corticotrophin or adrenocorticotrophic hormone, ACTH, which is secreted by the anterior pituitary gland. This secretion is regulated by corticotrophin-releasing factor, CRF, derived from the hypothalamus. Glucocorticoids have many actions but act like mineralocorticoids when occupying mineralocorticoid receptor sites, causing sodium retention and potassium loss. They are also thought to increase glomerular filtration rate.

SCHEMATIC REPRESENTATION OF THE GLUCOCORTICOID RECEPTOR GENE.



Reference. (Hollenberg. 1985)[19]

Section 5. Essential hypertension.

The term essential hypertension represents not a disease as such, but merely that section of the population having arterial pressures higher than an arbitrarily selected value and having no disease to which these pressures can be attributed.

The fundemental aetiology of essential hypertension remains largely unknown. Blood pressure is a quantitative trait showing a continuous variation in the population and hypertension represents the upper end of this distribution set by arbitrary limits. (In this study the 'cut off' point was taken as those people who had a diastolic blood pressure greater than 95 mm Hg.).

The first intimation that high blood pressure tended to be familial was indirect and stemmed from the 18th century observation of Morgagni (1761)[20]. The father of a patient who had died from cerebral haemorrhage had himself died of 'apoplexy' and although the connection of this and high blood pressure was not understood well, it gave rise to speculation that families had a disproportionate number of individuals whose death was due to strokes, caused by high blood pressure.

It was not until earlier this century that blood pressure could be measured by mechanical means, when the inflatable cuff of Riva-Rocci was attached to the sphygmomanometer of von Basch. Over the last 60 years a succession of surveys have measured the distribution of blood pressure in many

populations but not until 1954 , when the St. Mary's Study (Hamilton. 1954)[21], tried to understand the inheritance of hypertension by evaluating the distribution of blood pressure within families, with defined relationships, against those of the general population. However families tend to also share the same environmental factors that might contribute to raising blood pressure, but more defined studies using the results of human twin and adoption studies , (Feinleib. 1977)[22], (Annest.1979)[23], showed an identifiable genetic component suggesting that as much as 60% of diastolic and systolic blood pressure variability may be genetically determined. Using the hypertensive rat strain, as an animal model, it has been calculated that only a few genes are responsible for the control of blood pressure and that their contribution as individual genes can be determined. (Rapp.1983)[24].

Populations that have a high salt diet generally have higher blood pressures than those that have a low salt intake. (Intersalt. 1988)[25], and hypertension appears to be associated with the difficulty in excreting a salt load. (de Wardener. 1980)[26]. This inherent difficulty is more marked in black subjects (Luft. 1982)[27], in whom the prevalence of hypertension is also dependent on geographical regions. (Cruickshank. 1989)[28].

Section 6. Objective of study.

This study examines restriction fragment length polymorphisms using ' candidate genes ' that might be involved in causing high blood pressure, in three separate study groups, and assessing whether a correlation can be determined between genetic variants and high blood pressure and associated gene products.

Study group 1.

The first preliminary study was comparing RFLPs from two groups of subjects.

a) The hypertensive group, comprising of thirty eight patients attending the hypertensive clinic at St. Georges Hospital, Tooting. These subjects had diastolic blood pressures, before treatment, greater than 100mm Hg. on repeated measurements, with no evidence of secondary hypertension and with at least one known first degree relative with hypertension.

b) The control group, comprising of ninety eight blood donors with undefined blood pressure, none of whom were known hypertensives.

Study group 2.

The second study group analysed RFLPs and measured plasma ANP and aldosterone and urinary sodium from twenty subjects, with no known hypertension, randomly selected from the staff in the Child Health department at St. Georges Hospital,

Medical School. Blood pressure was measured after 5 minutes and 30 minutes of lying prone and 10 ml of blood was taken into E.D.T.A. tubes immediately after each measurement. A urine sample was collected after the 30 minute rest period.

Study group 3.

The third study group involved three hundred and eighty subjects aged 45-74 years, randomly sampled from the registers of two North London Health centres, who were participating in a cardiovascular project. These were split into three groups, of which white caucasians (n= 123) and black Afro-Caribbeans (n= 121) defined by three grandparents in the same ethnic group, were selected. (A third Gujerati group were not analysed.) Blood pressure was measured twice for each subject, by a trained observer, after sitting for at least 5 minutes. Fasting venous blood samples were taken and separated for DNA extraction and plasma renin assay. A subset of this population was selected, representing the upper and lower quintiles of diastolic blood pressure, for both ethnic groups, from both sexes, within each group and RFLPs and plasma active renin levels were measured.

CHAPTER TWO MATERIALS AND METHODS

ABBREVIATIONS AND FORMULAE

BSA	BOVINE SERUM ALBUMIN
$CaCl_2$	CALCIUM CHLORIDE
cDNA	COMPLEMENTARY DEOXYRIBONUCLEIC ACID
d-ATP	DEOXY-ADENOSINE TRIPHOSPHATE
d-CTP	DEOXY-CYTIDINE TRIPHOSPHATE
d-GTP	DEOXY-GUANINE TRIPHOSPHATE
d-TTP	DEOXY-THYMINE TRIPHOSPHATE
DMSO	DIMETHYL SULPHOXIDE
EDTA	ETHYLENE-DIAMINE-TETRA-ACETIC ACID
HCl	HYDROCHLORIC ACID
KCl	POTASSIUM CHLORIDE
LB	LURIA-BERTANI
$MgCl_2$	MAGNESIUM CHLORIDE
MOPS	N-MORPHOLINO-PROPANE-SULPHONIC ACID
NaCl	SODIUM CHLORIDE
Na_2HPO_4	DISODIUM HYDROGEN ORTHOPHOSPHATE
$NaH_2PO_4.2H_2O$	SODIUM DIHYDROGEN ORTHOPHOSPHATE
NaOH	SODIUM HYDROXIDE
RNA	RIBONUCLEIC ACID
SDS	SODIUM DODECYL SULPHATE
SSC	SALINE-SODIUM-CITRATE
STE	SALINE-TRIS-EDTA
TAE	TRIS-ACETATE-EDTA
TBE	TRIS-BORATE-EDTA
TE	TRIS-EDTA
TRIS	TRIS-HYDROXY-METHYL-AMINO-ETHANE-SULPHONIC ACID

Analysis of DNA sequences by Southern blotting analysis.

Restriction fragment length polymorphisms (RFLPs) were determined on DNA extracted from whole blood from test subjects, by digesting the genomic DNA with various restriction enzymes, separating the restricted DNA fragments on agarose gels by electrophoresis, transferring and immobilising the DNA from the agarose onto nylon membranes by Southern blotting, and hybridising with appropriate radioactive probes that were visualised by autoradiography.

Hybridisation probes.

C-DNA from genes that might be involved in the regulation of blood pressure were used as hybridisation probes and were obtained as DNA fragments in solution or transformed into various bacterial vectors. The PND was a genomic clone.

Candidate gene probes.

Human pro-natriodilatin (PND)

Human atrial natriuretic peptide (ANP)

Human renin

Human kidney kallikrein

Human glucocorticoid receptor

Human PND (pro-natriodilatin) genomic DNA, a 1 Kb full length fragment, was cloned into the Eco RI - Bam HI site of an undocumented plasmid JA110 and obtained as 1.5 ug of plasmid in 70% EtOh and was transformed into bacterial strain HB101.

Human ANP (atrial natriuretic peptide) cDNA, a full length 0.7 Kb fragment, was cloned into the Eco R1 site of pUC9, in an unfamiliar bacterial strain and obtained as a stab in culture medium.

Human renin cDNA, isolated from a human kidney cDNA library, was isolated as two contiguous Eco R1 fragments of 0.75 Kb (from the 5' end) and 0.70 Kb (from the 3' end) and individually subcloned into pUC9. They were obtained as purified plasmid DNA in TE buffer at a concentration of 50 ng/ µl and transformed into bacterial strain RRI.

Human kidney kallikrein cDNA, a full length 1.5 Kb fragment, was cloned into the Eco RI site of pUC and obtained as purified plasmid that was transformed into bacterial strain RRI.

Human glucocorticoid receptor cDNA, a 1.1 Kb fragment cloned into the Eco RI site of a pUC8 derivative, hGR1.2, was obtained as purified plasmid and transformed into bacterial strain RRI.

Transformation into competent cells.

A method based on Mandel and Higa (1970)[29] was used to introduce plasmid DNA into bacterial cells .

One colony from a plate of HB101, an E.Coli strain, was inoculated into 20 ml of LB broth and incubated at 37°C overnight with shaking. 5 ml of this overnight culture was added to 20 ml of LB broth and incubated at 37°C until an optical density reading of 0.5 was reached at absorbance wavelength 550 nm.

The relationship between optical density and the number of bacteria per ml. of culture varies from strain to strain.

HBI0I 1 OD_{550} = 0.5 (5 x 10^7 cells / ml.)

RR1 1 OD_{550} = 0.2 (5 x 10^7 cells / ml.)

These readings denote the optimum concentration for bacterial growth.

Cells were spun down at 4000 g at 0°C for 5 minutes, the supernatent discarded, and cells resuspended in 10 ml of ice-cold 10 mM Tris pH 7.3 + 50 mM $CaCl_2$.

The cell suspension was chilled on ice for 5 minutes and spun down again at 4000 g at 0°C for 5 minutes, the supernatent discarded, and cells resuspended in 2 ml ice-cold 10 mM Tris pH 7.3 + 50 mM $CaCl_2$, and once more spun down at 4000 g at 0°C for 5 minutes and the supernatent discarded.

200 µl of the cellular pellet was mixed with 100 µl of plasmid DNA solution (1 µg/ ml in TE buffer), and the cells incubated at 37°C for 2 minutes and at room temperature for a

further 10 minutes. 0.5 ml of LB broth was added and incubated at 37°C for 30 minutes.

2.5 ml of softened LB agar was mixed with the incubated cells and poured onto a plate containing bottom agar with the relevant antibiotic. 50 µg/ ml of ampicillin or 12.5 µg/ ml of tetracycline.

The plate was incubated overnight at 37°C and individual colonies picked out.

Characterisation of transformants.

The use of plasmids with genes for antibiotic resistance with host bacterial cells, plasmid - free, made easier identification of recombinant DNA.

The cDNA cloned fragment, was inserted into part of the ampicillin - resistant gene, inactivating the antibiotic sensitivity. Introduction of the recombinant plasmid into the host bacterium resulted in successful recombinants growing up on ampicillin LB plates.

The choice of plasmid determined the antibiotic and the restriction site used for insertion and, subsequently, excision.

The PND clone was received in plasmid with information only on the restriction enzymes used for insertion, and no indication as to whether antibiotic resistance had been modified.

A method for establishing successful recombinants, without using antibiotic resistance, was used.

20 µl of LB broth was pipetted into each well of a sterile microtitre plate and an individual colony inoculated into each.

The plate was covered and incubated at 37^{o}C overnight.

Using a 'hedgehog' type apparatus the colonies were transferred onto nitrocellulose membrane, previously wetted with molten agar. Alignment marks were made on the plate and membrane. The membrane was covered in plastic Saran wrap and incubated at 37^{o}C overnight and the microtitre plate was

covered and stored at 4°C.

After incubation the membrane was placed, colony side up, onto Whatman 3MM chromatography paper saturated with 10% SDS, for 3 minutes and then into the following solutions:-

0.5 M NaOH for 2 minutes, x 2

1.0 M Tris pH 7.5 for 5 minutes, x 2

0.5 M Tris pH 7.4 / 1.5 M NaCl for 3 minutes.

The membrane was wiped carefully with a tissue soaked in 2 x SSC / 0.1% SDS to remove any adhering agar and further washed in the same solution for 5 minutes. It was removed and placed onto a sheet of 3MM chromatography paper, air dried, and baked at 80°C for 2 hours, and stored covered at room temperature until use.

Hybridisation with PND probe

The genomic PND clone was redissolved in TE buffer at a concentration of 100 µg/ ml and radioactively labelled by Nick translation, using a commercially available kit supplied by Amersham. The details of which are explained in the section Nick translation method.

The standard protocol was followed.

The nitrocellulose membrane was handled gently to prevent damage and pre-washing and hybridisation was carried out in sealed polythene bags.

Hybridisation techniques are covered more fully under the section Hybridisation.

The membrane underwent the following :

Prewash for 1 hour at 42°C , followed by prehybridisation for 1 hour at 42°C and hybridisation, in fresh solution with denatured probe, overnight at 42°C.

After, the membrane was removed from the bag and washed 3 times for 5 minutes in 2 x SSC / 0.1% SDS and 2 times for 15 minutes in 0.1 x SSC / 0.1% SDS at room temperature.

The membrane was covered in Saran wrap and autoradiographed overnight. Details outlined in autoradiography section.

Positive clones were identified by alignment of the black spots on the autoradiograph to the corresponding colonies on the titre plate. These were grown up further in LB medium overnight at 37°C.

Pre-wash solution.

50 mM Tris pH 8.0	15 ml.
1 M NaCl	60 ml.
1 mM EDTA	3 ml.
0.1% SDS	3 ml.

Pre-hybridisation and hybridisation solution.

50% Formamide	50 ml.
5 x Denhardts solution	5 ml.
5 x SSC	25 ml.
0.3% SDS	3 ml.
Herring sperm DNA 10mg/ml	2.5 ml. (Denatured by heating at 95°C and placing on ice for 2 minutes.)

5 x Denhardts solution

0.5 g Ficoll (type 400), 0.5 g polyvinylpyrrolidone, 0.5 g BSA in 500 ml of deionised water.

Storage of recombinant DNA in host vectors.

All the bacterial strains containing the various plasmids were streaked out onto LB agar plates, containing the relevant antibiotic, in all cases this was ampicillin, incubated overnight at 37°C, to form single colony growth. The plates were tightly wrapped in clingfilm and stored, inverted to stop any condensation forming on the agar, at 4°C, and replated every 6 months.

For longer term storage, glycerol and DMSO stocks were made.

Glycerol stocks.

850 µl of an overnight culture and 150 µl of sterile glycerol were pipetted into a sterile 1.5 ml tube, capped , mixed thoroughly by vortexing, and stored at -20°C.

DMSO stocks.

500 µl of an overnight culture and 500 µl 15% DMSO solution were pipetted into a sterile 1.5 ml tube, capped , mixed thoroughly by vortexing and stored at -20°C.

Isolation of plasmid DNA.

The methods used involved growth of the bacteria, amplifying the plasmid, harvesting and lysis of the bacteria and purifying the plasmid. (Maniatis. 1982)[30]

Small scale-Mini plasmid preparation.

A single colony was picked from an LB agar plate with an inoculating loop and inoculated into 5 ml of LB medium containing the relevant antibiotic and incubated overnight at 37°C, with shaking.

1.5 ml of overnight culture was pipetted into a sterile 1.5 ml tube and spun at 4000 rpm for 5 minutes. The supernatent was discarded and the pelleted cells resuspended in ice-cold solution A.

Resuspension was carried out by pipetting up and down carefully with a pasteur pipette and incubated at room temperature for 15 minutes.

400 µl of freshly prepared ice-cold solution of 0.2 M NaOH and 1% SDS was added, mixed, by inverting the tube gently several times and incubated on ice for 10 minutes.

200 µl of Potassium acetate solution was added and the tube tightly capped and vortexed in an inverted position for 10 seconds and put on ice for 30 minutes.

The tube was spun at 4000 rpm at 4°C for 15 minutes and the supernatent pipetted into another 1.5 ml sterile tube. 500 µl of Isopropanol was added and mixed, and left at room temperature to precipitate the DNA. It was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatent carefully removed with a pipette and discarded. Any droplets left were adsorbed onto a paper tissue.

The pellet was resuspended in 40 µl of TE buffer pH 7.5 with the addition of 2 µl DNAse free RNAse (10 mg/ ml) and

incubated at 37°C for 30 minutes.

40 µl of Phenol / Chloroform solution 1:1 v:v was added, the tube capped and shaken gently until an emulsion formed.

It was centrifuged for 5 minutes at 4000 rpm at room temperature and the top aqueous layer pipetted off into a 1.5 ml tube. 40 µl of Chloroform / Isoamyl alcohol 24:1 v:v solution was added, the tube capped and again shaken gently and centrifuged as before. The top layer was removed into another 1.5 ml tube. 300 µl of Aristar grade ethanol was added , mixed gently and left at -70°C for 30 minutes.

The tube was centrifuged at 4000 rpm at 4°C for 15 minutes and the ethanolic supernatent pipetted off without disturbing the pellet, which was vacuum dried in a freeze drier and resuspended in 50 µl of TE buffer pH 7.5 containing 2 µl DNAse free RNAse (20 µg / ml).

Solution A.

50 mM Glucose

10 mM EDTA pH 8.0

25 mM Tris pH 8.0

4 mg Lysozyme (freshly added)

Potassium acetate solution.

60 ml 5 M Potassium acetate

11.5 ml Glacial acetic acid

28.5 ml distilled water

(3 M with respect to potassium - 5 M with respect to acetate)

Large scale Maxi plasmid prep.

A single colony was picked off of an LB agar plate and inoculated into a universal bottle containing 10 ml of sterile LB broth with 50 µl of ampicillin (10 mg / ml), and grown up overnight on an orbital shaker at 37°C.

100 µl of this culture was pipetted into another sterile flask containing 25 ml of LB broth with 125 µl of ampicillin, prewarmed, and grown up on an orbital shaker at 37°C until an aliquot taken gave an OD reading of 0.4-0.6 at wavelengh 600 nm, corresponding to late log phase in the bacterial growth curve.

25 ml of this culture was inoculated into 250 ml of pre-warmed LB broth containing 1.25 ml of ampicillin in a 500 ml flask and incubated at 37°C in an orbital shaker for approximately 2.5 hours until the OD_{600} reading reached 0.4. 1.25 ml of chloramphenicol solution (34 mg / ml in ethanol) was added and incubation continued overnight.

The addition of chloramphenicol was to promote plasmid amplification but little difference was noted between bacterial plasmid growth with or without it, so after the initial preparations, this step was left out.

Harvesting of cells.

The overnight culture was poured equally into two 300 ml pre-sterilised screw capped centrifuge bottles and centrifuged in a 6 x 300 ml angle head rotor on a MSE Hi-spin 21 centrifuge, at 6,500 rpm (4000g) for 15 minutes at 4°C.

The supernatent was discarded and 50 ml of ice-cold STE solution added to the pellet and resuspended gently, using a pasteur pipette, and centrifuged as before.

The supernatent was discarded and the pellet resuspended in 10 ml lysis buffer using a pasteur pipette.

The lysed cell solution was transferred to two 30 ml polypropylene centrifuge tubes and left to stand for 5 minutes at room temperature.

10 ml of freshly prepared 0.2 M NaOH / 1% SDS solution was added to each tube, capped, and inverted several times, then incubated on ice for 10 minutes.

7.5 ml of ice-cold potassium acetate solution was added, the tubes capped and quickly inverted several times and again incubated on ice for 10 minutes.

The tubes were centrifuged at 20,000 rpm, with the lids removed and parafilm covering the top of the tubes, for 20 minutes at 4°C. (The lids were removed because the tubes tended to warp at this speed and the lid would jam inside the tube).

The supernatent was removed into sterile 30 ml tubes and 0.6 times the volume of iso-propanol added.

Eg. 18ml of supernatent, 12ml iso-propanol.

The tube was left to stand at room temperature for 5 minutes for the DNA to precipitate and centrifuged at 12,000 g, which was 12,000 rpm using the MSE 8 x 50 ml angle head rotor, on the MSE Hi-spin 21 centrifuge, for 30 minutes at room temperature. At lower temperatures, the salt tended to precipitate out.

The supernatent was discarded and the pellet washed in 70% ethanol, recentrifuged at 12,000 g at room temperature for 5 minutes and the ethanolic supernatent discarded.

The pellet was dried under vacuum and dissolved in 6.0 ml of TE buffer pH 8.0.

The plasmid DNA was then purified.

<u>STE solution.</u>

10 mM Tris-HCl pH 8.0

100 mM NaCl

1 mM EDTA pH 8.0

Lysozyme 5 mg/ ml was added freshly just before use.

<u>Lysis buffer.</u>

50 mM Glucose

25 mM Tris-HCl pH 8.0

10 mM EDTA

<u>Potassium acetate solution pH 4.8.</u>

60 ml 5 M Potassium acetate

11.5 ml Glacial acetic acid

made up to 100 ml with sterile distilled water.

Solutions used in the growth of bacteria.

LB media (broth)

for 100mls

1 g Tryptone

0.5 g Yeast extract

0.5 g Sodium chloride

Bottom agar

for use in growing bacteria on plates.

1.5 g Agar in 100 ml LB broth.

Top agarose

for use in DNA lifts onto membranes.

0.7 g Agarose in 100 ml LB broth.

All chemicals used for growing up bacteria for cloning were of ultrapure specification rather than the normal microbiological grade.

Purification of closed circular DNA by centrifugation

6 ml of plasmid DNA solution was pipetted into a 10 ml MSE polypropylene centrifuge tube and 6 g of caesium chloride added, the tube capped and shaken gently until dissolved.

480 µl of ethidium bromide solution (10 mg / ml) was added to the caesium chloride solution in the tube and again capped and shaken gently.

The quantities used resulted in complete filling of the tube which prevented the tube from collapsing when centrifuged at high speed. When smaller quantities were used the tube had to be topped up with light paraffin oil.

Centrifugation was carried out in a 10 x 10 ml titanium angle head rotor on a MSE Prepspin 65 centrifuge over the weekend or a minimum time of 36 hours at 20°C at 45,000 rpm, with a gradual slow down, brake off system, in operation.

The tube was removed carefully and placed in an upright position. This was to re - orientate the bands from the diagonal position formed by the fixed angle head rotor.

After 10 minutes the tube was viewed, in a Chromata-vue cabinet under long wave UV light.

The lid was removed from the tube, to release the vacuum, and a disposable syringe and needle used to pierce the tube just below the plasmid band, the solution was drawn into the syringe carefully, to remove the plasmid band only.

(see figure 2.)

The syringe contents were emptied into 1.5 ml conical reaction tubes, and the syringe washed out with a minimum

amount of TE buffer, and the washings added to the tubes. An equal volume of butan-1-ol was added, the tubes capped, mixed vigorously, and centrifuged on a Beckman Microfuge at 1500 g, at 8000 rpm, for 3 minutes at room temperature.

Butan-1-ol was used to extract the ethidium bromide from the bottom aqueous layer into the top organic layer. The lower aqueous layer was removed, by using a fine pipette tip, and transferred to another 1.5 ml tube.This extraction procedure was repeated four more times or until all the pink colouration was removed from the aqueous phase. This was pipetted into pretreated dialysis tubing, clipped at each end, and dialysed against two changes of TE buffer pH 8.0.

The contents of the dialysis tube were measured into a 30 ml sterile tube and one-tenth volume of 3 M sodium acetate solution pH 5.2 was added followed by two times volume of ethanol and mixed. It was left to stand at room temperature to precipitate the plasmid DNA.

The tube was centrifuged at 5000 rpm for 10 minutes at room temperature and the supernatent removed carefully with a pipette. The pellet was washed in 70% ethanol to remove all traces of salt contamination and dried under vacuum.

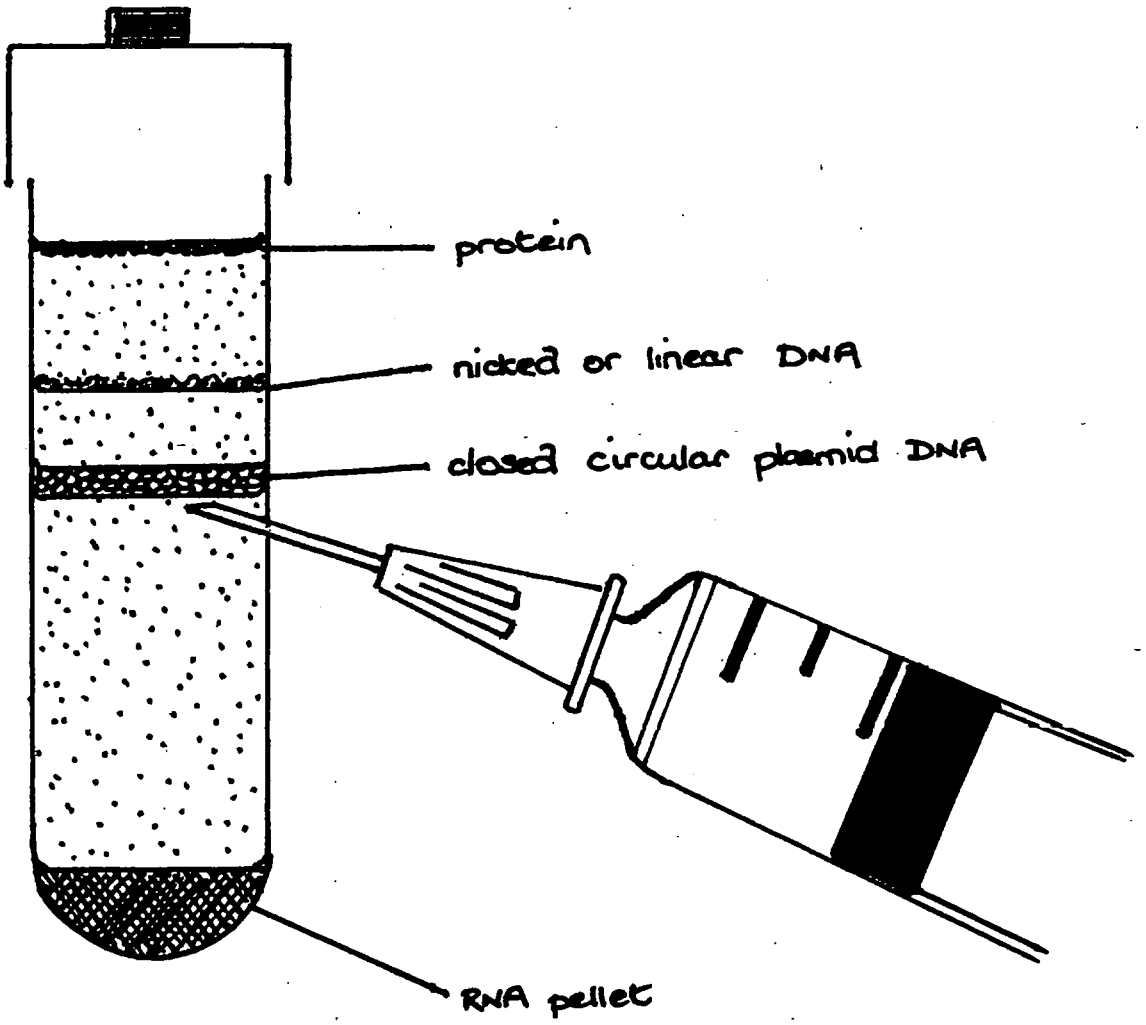
The DNA pellet was resuspended in 500 µl of TE buffer pH 8.0 with the addition of Dnase - free Rnase to a final concentration of 10 µg / ml.

Pre-treatment of dialysis tubing.

Dialysis tubing was cut to the suitable lengths and boiled up in a solution of 2% sodium bicarbonate and 1 mM EDTA for 10 minutes.The tubing was removed and washed several times inside and out with sterile distilled water. The lengths were put into deionised water in a screw capped jar and autoclaved, cooled and stored at 4^{o}C.

FIGURE 2.

REMOVING PLASMID DNA BAND AFTER CAESIUM CHLORIDE GRADIENT CENTRIFUGATION.



Alternative plasmid purification using Quiagen columns

Quiagen anion exchange resin columns (distributed by Hybaid) were brought onto the market in 1988 and were used for the later plasmid preparations.

An overnight 250 ml of culture was grown up, the bacterial cells spun down and the supernatent removed, as before.

The protocol from the Quiagen kit was then followed.

The bacterial pellet was resuspended carefully in 10 ml of the kit buffer P1.

10 ml of buffer P2 was mixed gently and incubated at room temperature for 5 minutes.

10 ml of buffer P3 was added ,the whole mixed gently and centrifuged at 10,000g for 30 minutes at 4^{o}C.

The Quiagen column was equilibrated using a syringe filled with 5 ml of buffer solution QB and pushed through at a rate of 5 ml / minute.

The supernatent was applied to the column using a syringe and pushed through at a rate of 2 ml / minute.

20 ml of buffer QC was applied and pushed through at a rate of 5 ml / minute.

A clean tube was inserted under the column and the plasmid DNA was eluted with 5 ml of buffer QF.

The DNA was precipitated with 0.8 volumes of isopropanol.

Composition of Quiagen buffers.

P1	50 mM Tris-HCl, 10 mM EDTA, 400 µg RNase A / ml (pH 8.0)
P2	200 mM NaOH, 1% SDS
P3	2.55 M potassium acetate (pH 4.8)
QB	750 mM NaCl, 50 mM MOPS, 15% ethanol (pH 7.0)
QC	1000 mM NaCl, 50 mM MOPS, 15% ethanol (pH 7.0)
QF	1200 mM NaCl, 50 mM MOPS, 15% ethanol (pH 8.0)

The Quigen anion-exchange resin was covalently linked to a silica gel base with a hydrophilic surface coating preventing non-specific adsorption. The bacterial cells were lysed (steps 1-3) and the lysate adsorbed onto the column (steps 4-5). The impurities were washed off (step 6) and the DNA eluted off (step 7). The binding, washing and elution conditions depended on the salt concentration and buffer pH, which had been carefully worked out by the manufacturers, so the volumes of buffers used were critical.

This second method of plasmid purification was found to be much faster and gave similar yields of plasmid DNA, (500-1000 µg) and was used in preference to the first.

Spectrophometric estimation of DNA concentration.

1 µl of the plasmid DNA solution was pipetted into a 1.5 ml tube and 990 µl of water added and vortexed.

The UV / Visible scanning spectrophotometer (Philips) was set up to scan from 240 nm to 300 nm and to give readings at 260 nm and 280 nm. A 1 ml micro quartz cell was filled with water, placed in the cell holder and used to set up the machine zero and sample blank. The same cell was emptied, dried and the DNA sample pipetted in, placed back into the cell holder and the sample scanned. A printer was attached to the spectrophotometer and a hard-copy of the scan and readings obtained.

DNA, RNA and oligonucleotides were quantitated by calculating the concentration of nucleic acid in the sample read at 260 nm.

An OD of 1 is equivalent to:

50 µg / ml for double stranded DNA.

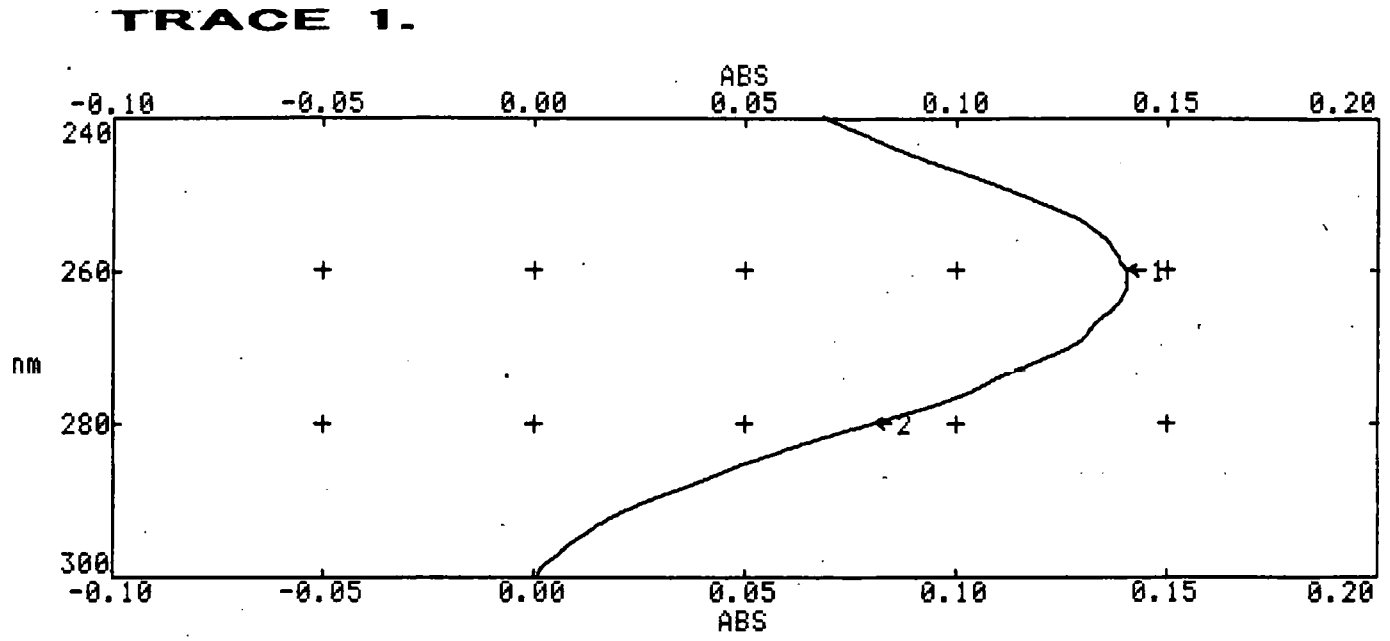
40 µg / ml for single stranded DNA and RNA.

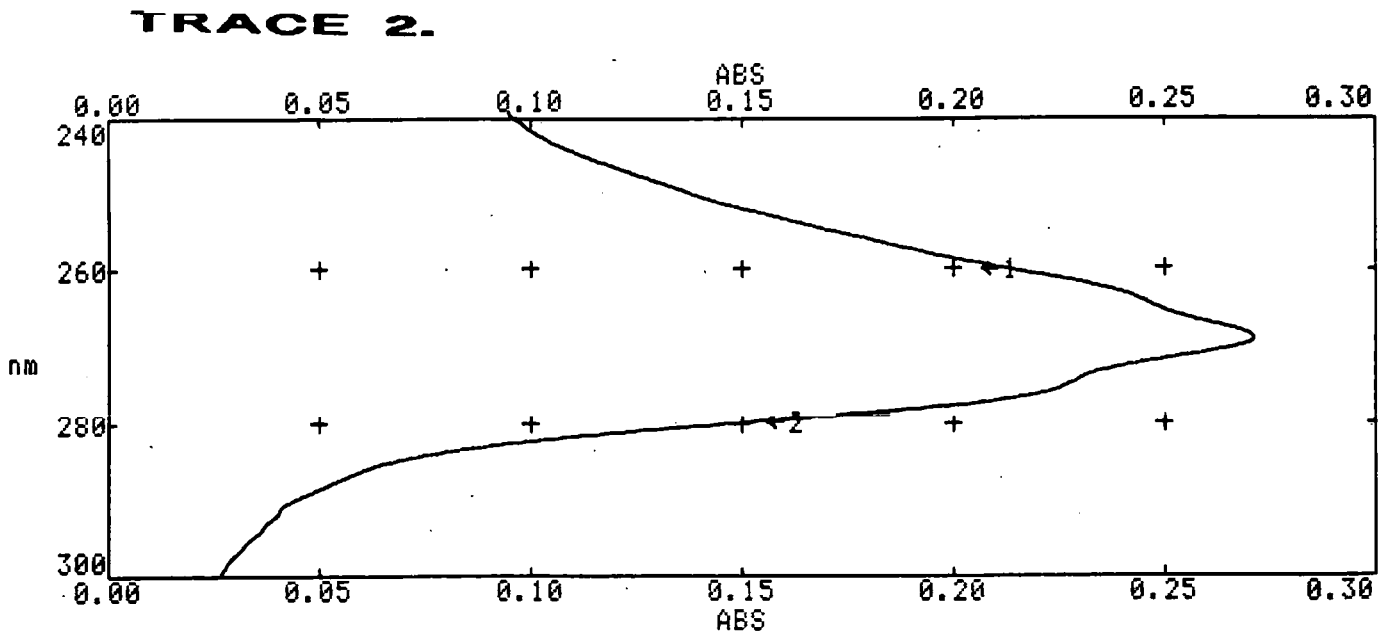
20 µg / ml for oligonucleotides.

The sample was also read at 280 nm, as protein contaminant absorbed at this wavelength, and a ratio of OD_{260} / OD_{280} provided an estimate for the purity of the nucleic acid.

A pure preparation gave a ratio of 1.8.The sample was scanned from 240 nm to 300 nm to evaluate the purity of the sample. A smooth curve represented an uncontaminated sample. Phenol absorbs at 270 nm so any contamination showed up as a small peak or less smooth curve. (Figure 3.).

FIGURE 3. SPECTROPHOTOMETRIC SCANS OF PLASMID DNA.





Trace 1. DNA scan showing non - contaminated DNA

Trace 2. DNA scan showing phenol contamination in DNA

Calculation for estimation of DNA concentration.

OD reading at 260 nm x 50 (for ds DNA) x dilution factor.

= µg / ml.

Example

DNA sample in 500 µl TE buffer.

10 µl of sample diluted with 990 µl of water.

OD reading at 260 nm = 0.28

x 50 (factor for ds DNA)

x 100 (factor for dilution)

= 1400 µg / ml.

Total volume was 500 µl, therefore total amount of DNA in sample was 700 µg.

Ratio of reading at 260 nm and 280 nm.

Reading at 260 nm = 0.28

Reading at 280 nm = 0.158

Ratio 260 / 280nm = 0.175

Therefore the sample was taken as being a clean preparation.

Excision and recovery of insert DNA from plasmid DNA.

100 µl of purified plasmid was restricted with the relevant restriction enzyme.

A normal digest would consist of:

100 µl plasmid DNA (140 µg from the previous example)

10 µl 10 x restriction enzyme buffer

5 µl restriction enzyme

Incubated at 37°C for a minimum of 1 hour or overnight.

The length of time for digestion was not critical as contamination by exonucleases had been minimised.

The restricted plasma sample was then separated using gel electrophoresis.

Recovery of insert DNA from plasmid using low melting agarose gels in a mini-gel electrophoresis system.

The standard method for separating and purifying DNA fragments is described in the gel electrophoresis section.

This method of excising insert DNA out of plasmid DNA substitutes normal agarose for a low melting or gelling agarose developed by Weislander (1979)[31] and is used in a mini-gel electrophoresis system. (see figure 4.)

Low melting point agarose has hydroxyethyl groups introduced into the agarose molecule and this substitution causes the agarose to gel at 30°C and melt at 65°C. To aid setting, the gel was poured and set at 4°C , and electrophoresis carried out at a low voltage (5 volts / cm), to prevent the gel from heating up and melting.

The separation of the insert DNA from the plasmid DNA was carried out in 1% low melting point agarose in 1 x TBE buffer and ethidium bromide added to the gel at a final concentration of 0.5 µg / ml. The capacity of the mini- gel was 30 ml and autoclave tape was used to seal the two ends of the gel mould.

The teeth of the gel comb were taped to attain a long well that would hold 100 µl of the plasmid digest. 10 µl of DNA size marker solution was pipetted into the adjoining well to determine band sizes on visualisation of gel.

After electrophoresis, the insert band was localised by viewing the gel under long wave UV 300-360 nm.(See figure 5.). The band was cut out from the gel using a scalpel blade,

taking care to trim the amount of excess agarose to a minimum, and put into a pre-weighed sterile glass tube. Long wave UV was used to localise the DNA bands to minimise the damage by short wave UV, which tended to 'nick' the DNA into shorter pieces.

The weight of the insert in agarose was determined and 3 x volume of sterile deionised water added.

Example

Wt. of insert in agarose = 1 g

Volume of water added = 3 ml

Total volume melted = 4 ml

The tube was capped and the DNA denatured by heating for 7 minutes in a boiling waterbath, and aliquots containing approximately 100 ng of insert were pipetted into sterile 1.5 ml conical reaction tubes and stored at -20°C.

Rough estimation of insert concentration.

Plasmid + insert = 140 µg

Plasmid size = 3.5 Kb.

Insert size = 1.0 Kb.

Therefore the insert was taken as being about a quarter of the total.

Excised insert = 35 µg in 4.0 ml agarose solution.

Therefore 100 ng = 11 µl of agarose solution.

This was used directly in the oligo-labelling procedure.

FIGURE 4. MINI GEL ELECTROPHORESIS.

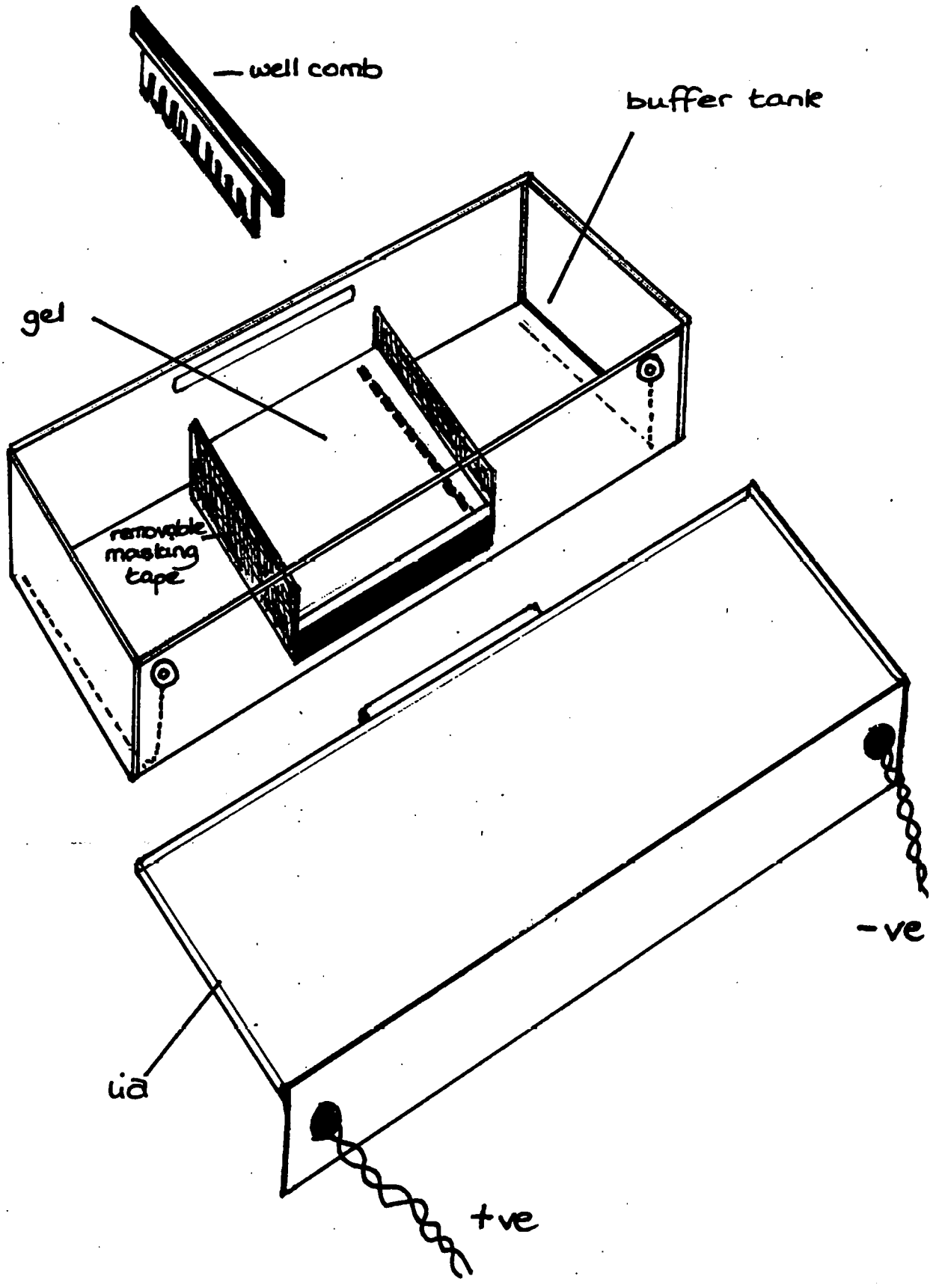
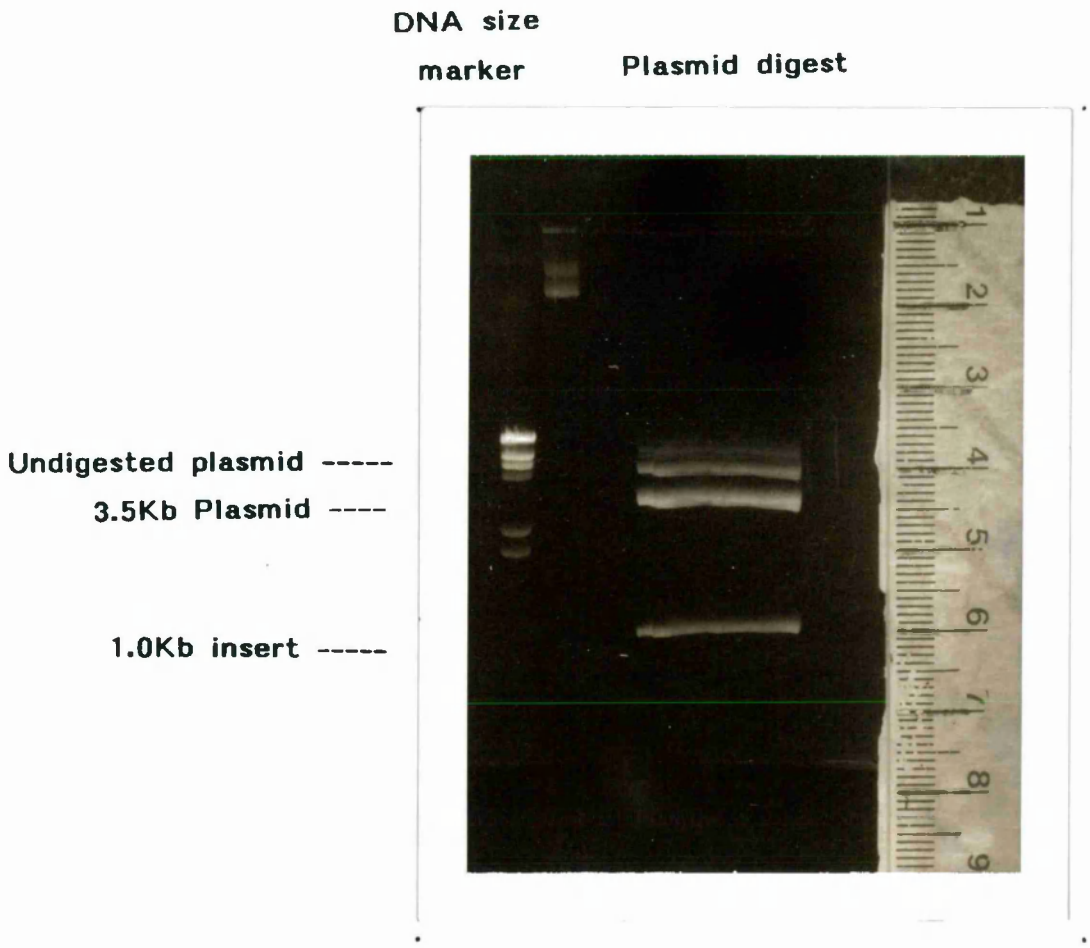


FIGURE 5. POLAROID PHOTOGRAPH OF PLASMID AND INSERT DNA.



Recovery of DNA from agarose gel by electroelution.

An alternative to using the low melting point agarose, was to separate the insert DNA from the plasmid DNA using normal 1% agarose gel electrophoresis and electroeluting insert DNA out of agarose and into dialysis tubing.

This method was first described by McDonnell (1977)[32]

The restricted plasmid was electrophoresed, the insert band cut out from the gel under long-wave UV, and the block of agarose gel, containing the insert, put into prepared dialysis tubing, clipped at one end and 0.5 x TBE buffer added to fill the tube. It was necessary to use forceps and gloves to handle the tubing to prevent nuclease contamination.

The excess buffer was removed and the tubing clipped, avoiding trapping air bubbles, so that the gel was in constant contact with the minimum amount of buffer.

The tubing was immersed in a shallow layer of 0.5 x TBE buffer in an electrophoresis tank, and a current passed for 2 hours at 100 volts. This electroeluted the DNA out of the gel and onto the inner wall of the tubing. The polarity was reversed, for 2 minutes, to release the DNA back into the buffer.

The tubing was unclipped at one end and the buffer carefully removed and measured, with a pipette, into a 1.5 ml conical reaction tube.

One tenth volume of 3M sodium acetate solution (pH 5.2) and two times volume of ethanol was added, the tube inverted

several times and left at room temperature for 5 minutes to precipitate the DNA.

The tube was centrifuged in a Microfuge (Beckman) at maximum speed equivalent to 8,000g for 10 minutes.

The supernatent was removed with a pipette, the pellet washed with 70% ethanol, and centrifuged again, for 5 minutes.

The supernatent was removed and the DNA pellet was dried under vacuum.

The pellet was resuspended in 100 µl of TE buffer pH 7.5 and the DNA concentration estimated using the spectrophotometric method described earlier, and additional TE buffer added to make a final concentration of 0.2 µg / ml.

25 µl aliquots (50 ng DNA) were pipetted into sterile 1.5 ml conical reaction tubes and stored at -20°C.

The DNA was aliquoted to prevent any deterioration caused by constant freezing and thawing of a larger volume.

All the quantities stated were for labelling by the random primer method. The concentration of DNA needed for the Nick translation method was higher at 200 µg / ml.

Radioactive labelling of DNA insert.

All radioactive procedures were carried out in a designated radioactive area of the laboratory and all safety measures were adhered to, including wearing protective coats, gloves and glasses and carrying out all manipulations on trays behind perspex screens.

Two methods of incorporating radioactive nucleotides into the DNA were used.

Both were kits manufactured by Amersham International and the standard protocol was followed.

A non - radioactive method of labelling the insert DNA was also tested using a kit from Betheseda Research Laboratories.

Radioactive labelling using the Nick translation kit.

The method was based on work published by Kelly (1970)[33]
In the reaction, the nucleotide sequence of the purified DNA was renewed, without increased synthesis. The DNA was nicked by DNase I and this formed the loci for the catalytic enzyme DNA polymerase to replace existing nucleotides with replacement nucleotides, of which one, dCTP was radioactive. The amount of radioactivity incorporated depended on the extent of renewal of the nucleotides and the specific activity of the radiolabelled dCTP.

Solutions obtained in the kit were:-

Nucleotide buffer solution (1)

100 µM dATP

100 µM dGTP

100 µM dTTP

in Tris-HCl pH 7.8, magnesium chloride , 2-mercaptoethanol.

Enzyme solution (2)

5 U DNA polymerase I

100 pg DNase I

in Tris-HCl pH 7.5, magnesium chloride, glycerol , bovine serum albumin

The radiolabelled dCTP was supplied from Amersham or NEN as

alpha-^{32}P deoxycytidine 5' triphosphate

in 10 mM Tricine pH 7.6 at 10 mCi / ml.

Specific activity = 3000 Ci / mmol.

The DNA solution was denatured by heating for 2 minutes in a boiling waterbath and immediately put on ice. All procedures were carried out on ice.

The following solutions were added, in order, in a 1.5 ml polypropylene microcentrifuge tube.

5 µl	DNA solution (1 ug)
10 µl	Nucleotide buffer solution (1)
5 µl	^{32}P-dCTP
25 µl	Water
5 µl	Enzyme (2)

The solutions were mixed by pipetting up and down and placed in a constant temperature bath at 15°C for a minimum of 2 hours, maximum of 6 hours.

Removal of unincorporated label using a Sephadex G50 column.

A pasteur pipette was plugged with siliconised glass wool and a slurry of Sephadex G50 medium grade in TE buffer pH 7.5 pipetted in, until a packed column was formed up to 1 cm from the top of the pipette.

The Sephadex had been pre-swollen by adding 3 x volume TE buffer to the dry Sephadex in a screw-capped jar, autoclaved and stored at 4°C until used.

The column was equilibrated with 2 ml of TE buffer. Because of the small diameter of the glass tube, the buffer remained on the column, and it was not necessary to clamp the tube to prevent it drying out.

1.5 ml tubes were placed in a rack under the column to

collect the waste eluate. 1 µl of 5% Dextran blue solution was added to the reaction mixture and together loaded directly onto the column.

100 µl aliquots of TE buffer were used to elute the DNA and the blue dye ran as a visible band down the column and coincided with the labelled DNA. The blue fraction, normally 600 µl, was collected in a screw capped 1.5 ml tube and 6 µl (one-hundredth) aliquot taken for counting the radioactivity incorporated.

The free labelled nucleotides remained on the column.

The labelled DNA fraction was denatured by putting the capped tube on a polystyrene float in a boiling waterbath for 7 minutes and then on ice for 5 minutes.

Scintillation counting.

The amount of radioactivity incorporated was estimated.

The 6 µl aliquot of the labelled DNA was pipetted into a 1.5 ml conical reaction tube and the whole tube placed inside a 20 ml scintillation vial. It was not necessary to add any scintillation fluid as the high energy beta emission from the ^{32}P was counted by Cerenkov method.

This method works on the principle that fast moving charged particles travelling through a transparent medium (in this case, polyethylene) radiate electromagnetic radiation picked up by the photomultiplier tube in a liquid scintillation spectrometer.

The energy is comparable to that of ^{3}H and the counting window was set up to measure this range.

6 µl Aliquot = 19×10^{4} counts per minute (cpm)

therefore 600 µl = 19×10^{6} cpm

To hybridise one membrane filter a minimum of 3×10^{6} cpm was used. This ensured dense black bands on the autoradiograph, after an overnight exposure.

The excess labelled DNA was stored at -20°C and used within a week. On reuse, it was necessary to denature the DNA again in a boiling waterbath for 2 minutes and on ice for 5 minutes.

The half-life of ^{32} P was 14 days, so probes were used within the first 2 weeks of labelling, to ensure strong signal bands on the autoradiographs.

Using this 'rule of thumb' guide was adequate to indicate the signal strength of the labelled probe.

The more accurate formula, from the Amersham booklet, was not used as the actual concentration of DNA could not be accurately determined.

Radioactive labelling using the Oligonucleotide multiprime kit.

This kit was obtained from Amersham International and the method used was based on that of Feinberg and Vogelstein (1983)[34]. Random sequence hexanucleotides were introduced to prime DNA synthesis at various sites along the length of the denatured DNA template, the DNA insert. The 'Klenow' fragment of DNA polymerase I was used to ensure that 5'- 3' exonuclease activity was absent, and that incorporated labelled nucleotides were not removed as monophosphates. Strand displacement and subsequent recopying of the DNA resulted in the newly synthesised DNA being in excess of the original amount of DNA, leading to more labelled nucleotide being incorporated and therefore 'hotter' probes providing stronger signals on autoradiographs. This method was used in preference to 'Nick translation' as less DNA was needed, the specific activity of the probe was higher, and that the oligolabelling reaction worked when the DNA was in low melting point agarose.

The standard protocol was followed, exept that the amount of labelled d-CTP used, was less than stated in the manufacturers instructions.

Multiprime buffer solution. (1)

dATP, dGTP and dTTP in a concentrated solution of:

Tris-HCl pH 7.8

Magnesium chloride

2-Mercaptoethanol

Primer solution. (2)

Random hexanucleotides in an aqueous solution containing nuclease-free BSA.

Enzyme solution

1 Unit / µl DNA polymerase I Klenow fragment in 50 mM potassium phosphate pH 6.5, 10 mM 2-mercaptoethanol and 50% glycerol.

The DNA in low melting point agarose was denatured by heating in a boiling waterbath and then left to cool down in a 37°C incubator for 10 minutes.

The following were added, in order, into a 1.5 ml tube:

25 µl DNA solution in low melting point agarose.
10 µl Multiprime buffer (1)
5 µl Primer (2)
6 µl Water
2 µl Enzyme solution
2 µl ^{32}P-dCTP

The solutions were mixed and incubated at 37°C for a minimum of 1 hour or left overnight at room temperature.

The unincorporated radioactivity was separated, on a G50 Sephadex column, and the amount of radioactivity incorporated calculated as before for the Nick translation system. DNA was collected in a screw capped 1.5 ml tube and denatured by heating for 7 minutes in a boiling waterbath and immediately placed on ice.

The labelled probe was then added directly into the hybridising solution.

Non - radioactive labelling of DNA .

A non - radioactive method for labelling the DNA insert was assessed. This was based on biotinylated nucleotide incorporation into the DNA using the standard Nick translation system and colourimetric detection of the hybridised filter with streptavidin - alkaline phosphatase conjugate and dyes.(BRL. Nucleic acid detection kit)

The advantages of using non - radioactive probes were that manipulations were carried out without having the safety problems involved when using high energy beta emmitters as ^{32}P and also that biotinylated labelled probes were stable up to two years if stored at -20^{o}C.

An initial experiment was carried out to compare the signal intensity of the colourimetric verses the radioactive labelled 'Y'probe, a high tandem repeat sequence probe specific for the 'Y' chromosome, on a duplicate filter of genomic digests of female and male subjects.

The comparative results from the two methods are shown in table 1.

The non - radioactive method was not used after these initial experiments because of the low probe signal obtained, the inability to reprobe a filter and not being able to have a long-term record of the results.

TABLE 1.

COMPARATIVE USE OF RADIOACTIVE AND NON-RADIOACTIVE PROBES.

RADIOACTIVE PROBE	BIOTINYLATED PROBE
High intensity black bands on autoradiograph with clear background.	Just detectable purple bands on highly coloured background.
Hybridisation carried out in non-specific plastic bags or tubes at 65°C.	Hybridisation carried out in special polyester bags that do not adsorb biotin.
Membranes could be stripped and reprobed.	The dyes used were irreversibly bound to the membrane so reprobing was not possible.
Membranes washed down in two solutions and monitored using a Geiger-Müller tube.	Membranes washed down twice in three solutions and blocked in another to prevent highly coloured background.
A permanent record of the results, the autoradiograph, could be kept and re-examined.	The coloured bands faded so unable to keep permanent records.

Extraction of DNA from whole blood.

The method used was adapted from the method by Gross-Bellard (1972)[35] and Bertelson (1988)[36] and was based on cell lysis followed by deproteinization and extraction of DNA.

Whole blood was taken by venupuncture from test subjects, collected in 10 ml EDTA tubes and either stored whole at -20°C, or red and white cells separated from the plasma by centrifugation, at 2000 rpm for 5 minutes at 4°C and the plasma removed into another tube, and both fractions stored at -20°C until further analysis.

Whole blood or blood cells were thawed on ice and poured into 30 ml disposable polypropylene centrifuge tubes and washed out twice, with ice-cold STE and the volume made up to 25 ml. The tube was capped and inverted several times and stored on ice for 5 minutes.

Centrifugation was carried out at 4°C at 10,000 rpm for 10 minutes using a 8 x 50 ml angle head rotor on a MSE Hi-spin 21 centrifuge.

The supernatent was carefully poured off from the pellet and the tube inverted onto a paper towel to drain off excess supernatent.

5 ml of ice-cold lysis solution was pipetted into the tube and the pellet resuspended using a pasteur pipette.

100 µl of 10% SDS solution was added followed by 100 µl of Proteinase K solution. The tube was capped, mixed and left overnight at 37°C or 2 hours at 68°C , until all solid matter was digested. The tubes were cooled to room temperature and

5 ml of Phenol added, the tubes capped, and mixed gently by inverting the tube several times and centrifuged at 20°C at 10,000 rpm for 5 minutes. The top layer was removed, using a bent pasteur pipette into another 30 ml tube. 5 ml of Chloroform was added, the tube capped, mixed gently and again centrifuged at 20°C at 10,000 rpm for 5 minutes. The top layer was removed, using another bent pasteur pipette, into another 30 ml tube.

500 µl of 3 M sodium acetate was pipetted into the tube, and mixed, followed by 11 ml of ethanol. The DNA was precipitated by rotating the tube in a circular motion to 'spin out' the fibrous DNA into a single mass. A cloudy solution or small particles in the tube indicated that the DNA had degraded and these were discarded. The DNA was removed using a disposable inoculating loop and excess liquid removed , by touching the loop onto the side of the tube.

The pellet was transferred into a 1.5 ml reaction tube containing 300 µl of TE buffer pH 7.5 and dissolved on a rotating mixer, at room temperature, overnight.

To minimise the activity of endogenous nucleases it was essential that procedures were carried out quickly, on ice and precautions taken against shearing by gentle mixing during the extraction.

Sometimes the organic phase would not separate cleanly from the aqueous phase and this was normally due to a high concentration of DNA or cellular debris in the aqueous phase. In these instances a further 5 ml of TE buffer was added and

re-extracted with the organic solvent.

Redistilled phenol was used to extract the proteins from the nucleic acids and the high purity was necessary to stop breakdown or crosslinking of the DNA. 8-Hydroxyquinoline was added to the phenol as an anti-oxidising agent, an inhibitor of RNase activity and a weak chelator of metal ions. The yellow colour acted as a convenient way of identifying the organic phase.

Chloroform removed any phenol and lingering proteins from the nucleic acid preparation. The addition of iso-amyl alcohol prevented foaming and facilitated the separation of organic and aqueous phases. DNA samples that showed bad restriction enzyme digests after separation and visualisation on electrophoresis gels, were re-extracted with chloroform, reprecipitated with ethanol, the pellet washed in 70% ethanol, dried under vacuum and re-dissolved in TE buffer as before.

Re-extraction removed contaminants, including proteinase k, phenol, excess protein and high concentrations of salt.

Solutions used:

STE
0.1 M NaCl
0.01 M Tris-HCl pH 7.5
0.001 M Sodium EDTA

LYSIS BUFFER
0.01 M Tris-HCl pH 8.0
0.40 M NaCl
0.002 M Sodium EDTA

PROTEINASE K

10 mg / ml solution

Stored at -20°C

SDS

10% solution

100 g sodium dodecyl sulphate in 1 litre of distilled water.

PHENOL

Liquified Phenol obtained in highest purity grade. (Rathburn chemicals) equilibrated and stored under 0.1 M Tris-HCl pH 8.0 with 0.1% 8-Hydroxyquinoline added.

Stored at 4°C

CHLOROFORM

A mixture of chloroform and isoamyl alcohol 24:1 v/v.

3M SODIUM ACETATE

408 g sodium acetate.$3H_2O$ in 800 ml deionised water. pH adjusted to 5.2 with glacial acetic acid and made up to 1 litre with water.

Solution autoclaved.

ETHANOL

Highest purity grade (absolute) Ethanol

TE BUFFER

pH 7.5

10 mM Tris-HCl pH 7.5

1 mM EDTA pH 8.0

pH 8.0

10 mM Tris-HCl pH 8.0

1 mM EDTA pH 8.0

Solutions autoclaved.

1 M TRIS

121.1g Tris base dissolved in 800ml deionised water and the pH adjusted with concentrated HCl.

pH 7.5 approx 65ml conc HCl

pH 8.0 approx 42ml conc HCl

and made up to 1 litre with deionised water.

Solution autoclaved.

0.5 M EDTA

186.1g of di-sodium ethylene diamine tetraacetate-$2H_2O$ dissolved in 800ml deionised water and the pH adjusted to 8.0 with 40% NaOH solution and made up to 1 litre with deionised water.

Solution autoclaved.

Spectrophotometric estimation of DNA in blood samples.

10 µl of the DNA solution was pipetted into 990 µl of water and readings taken at wavelengths 260 nm and 280 nm as described in the section on spectrophotometric estimation of DNA concentration.

The samples were diluted to give a final concentration of 300 µg / ml.

A simplified method for calculating the dilution factor was used.

All samples were made in an initial volume of 300 µl.

The reading off the spectrophotometer was multiplied by the dilution factor (100) and the DNA factor (50) to give a concentration in µg / ml.

This figure would be the same as the final volume to make the sample to 300 µg / ml.

Example :

Reading from spec. at 260nm	=	0.15
x 100 (dilution factor)	=	15
x 50 (DNA factor)	=	750 µg / ml (conc. in 300 µl)
Final volume for 300 µg / ml	=	750 µl
Initial volume	=	300 µl
Dilution volume	=	450 µl

The DNA samples were labelled and stored at $4^{o}C$.

Restriction endonucleases.

Restriction endonucleases are enzymes that have been isolated from different bacterial strains, hence their abbreviated names, that recognise short DNA sequences and cut double stranded DNA at specific sites within or adjacent to the recognition sequence.

For example: EcoRI isolated from Escherichia coli RY13

Recognition sequence

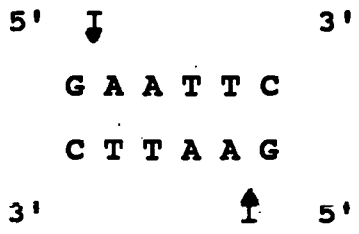


TABLE 2.

Recognition sequences and reaction conditions for enzymes used.

RE	Recognition sequence	Buffer*	Temperature
BamHI	G GATCC	Green	37°C
Bgl I	GCCNNNN NGGC	Green	37°C
Bgl II	A GATCT	Red	37°C
EcoR I	G AATTC	Black	37°C
Hae III	GG CC	Green	37°C
Hha I	GCG C	Black	37°C
Hinc II	GTPy PuAC	Red	37°C

* Colour coded. See page 69.

Hind III	A AGCTT	Black	37°C
Msp I	C CGG	Yellow	37°C
Nco I	C CATGG	Blue	37°C
Pst I	CTGCA G	Red	37°C
Pvu II	CAG CTG	***	37°C
Sca I	AGT ACT	Red	37°C
Taq I	T CGA	Red	65°C
Xho I	C TCGAG	Blue	37°C

C = Cytosine G = Guanine A = Adenine T = Thymine

Py = Pyrimidines (C or T) Pu = Purines (G or C)

N = Any nucleotide base

Restriction endonuclease assay buffers.

At the beginning of the project the restriction endonucleases were obtained from different suppliers and were of varying preparative quality. Assay buffer solutions were prepared for each individual enzymes optimum reaction condition, mainly based on ionic strength and correct pH and all restriction endonucleases require magnesium as a cofactor.

This led to many of the initial enzyme digests of genomic DNA showing incomplete digestion and causing many spurious bands, after Southern analysis, on the autoradiographs.

This problem was minimised when the suppliers sent with each enzyme, an optimised assay buffer solution. It was found that the buffer solutions obtained from Anglian Biotech covered the whole range of enzymes used and were colour coded for each.

Assay buffer solutions

Molarities given as final concentration in assay.

Yellow

6 mM Tris Hcl pH 7.5
6 mM $MgCl_2$
10 mM KCl
1 mM DTT
100 µg / ml BSA

Green

10 mM Tris HCl pH 8.0
10 mM $MgCl_2$
50 mM KCl
1 mM DTT
100 µg / ml BSA

Red

10 mM Tris HCl pH 8.0
10 mM $MgCl_2$
100 mM KCl
1 mM DTT
100 µg / ml BSA

Black

20 mM Tris HCl pH 8.0
10 mM $MgCl_2$
50 mM NaCl
50 mM KCl
1 mM DTT
100 µg / ml BSA

Blue

10 mM Tris HCl pH 8.0
10 mM $MgCl_2$
150 mM KCl
1 mM DTT
100 µg / ml BSA

*** PVU II

10 mM Tris Hcl pH 7.4
60 mM NaCl
10 mM $MgCl_2$
1 mM DTT
100 µg / ml BSA

Stock buffer solutions

1 M Calcium chloride

147 g $CaCl_2$ -$2H_2O$ in 1 litre deionised water

Solution autoclaved

1 M Dithiothreitol (DTT)

15.45 g DTT in 100 ml of sterile deionised water

Solution filter sterilised and stored at -20°C

1 M Potassium chloride

74.6 g KCl dissolved in deionised water

Solution autoclaved

1 M Magnesium chloride

20.3 g $MgCl_2.6H_2O$ dissolved in 100 ml of deionised water

Solution autoclaved

Bovine serum albumin (BSA)

10 mg BSA (fraction V) dissolved in 1 ml of sterile deionised water

Filter sterilised and stored at -20°C

Digestion of genomic DNA with Restriction Endonucleases.

The amount of genomic DNA to be fully digested by the restriction endonuclease was determined by restricting 2 µg, 5 µg, 10 µg and 20 µg of DNA for 2 hours or overnight with 10 units of appropriate enzyme and carrying out Southern blot analysis.

Amount of DNA restricted		Probe signal	Digestion
2 µg	2 hours	-	✓
2 µg	Overnight	-	✓
5 µg	2 hours	✓	✓
5 µg	Overnight	✓	✓
10 µg	2 hours	✓	✓
10 µg	Overnight	✓	✓
20 µg	2hours	too intense	partial
20 µg	Overnight	too intense	partial

From these results the amount of genomic DNA used in the Southern blot analysis was between 5 - 10 µg.

The minimum amount of time for genomic DNA digestions was 2 hours, however most were performed overnight or longer without any adverse degradation of the DNA.

The quality of the digestion was ascertained from the polaroid picture taken of the agarose gel after digestion and separation.(See figure 6.)

Restriction endonuclease assay.

Into a 1.5 ml reaction tube the following solutions were added in order:-

For a 50 µl volume digest.

5 µl 10 x enzyme reaction buffer

X µl sterile deionised water

20 µl genomic DNA solution (conc. 300 µg / ml)

2-5 µl enzyme

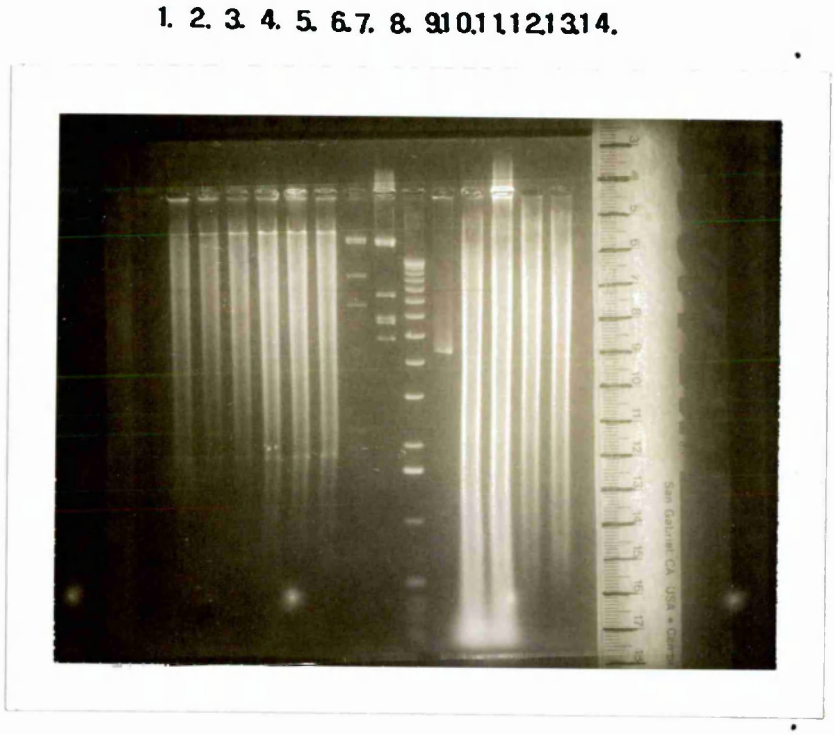
This was incubated overnight in a 37°C oven.

Enzymes that were known to be difficult to digest genomic DNA were added in two aliquots, the second aliquot two hours after the first. The addition of 1 µl of 1% spermidine solution sometimes aided the digestion by making the restriction sites along the DNA more available, however in low salt buffer conditions the DNA tended to precipitate out.

10 µl of digest was run on the mini-gel electrophoresis system to check that DNA digestion was complete.

FIGURE 6.

POLAROID PHOTOGRAPH SHOWING EXAMPLES OF DNA DIGESTED WITH RESTRICTION ENZYMES.



LANES 1 - 6 PARTIAL DIGEST (DNA REMAINING IN LOADING WELL)

LANES 7 - 9 DNA SIZE MARKERS

LANE 10 BAD DIGEST

LANES 11, 12 DNA SHOWING DEGRADATION

LANES 13, 14 GOOD DIGEST

Thé amount of enzyme needed to give a good digestion varied from one enzyme to another. Most were supplied at a concentration of 10 units / µl.

The Unit definition is:

1 Unit of enzyme will digest 1 µg of DNA in 50 µl of assay buffer in 1 hour at 37°C.

The minimum of 10 units (1 µl) was used initially but for some enzymes it was necessary to increase this up to 50 units.

The enzymes were obtained in glycerol solution and it was necessary to keep the concentration of glycerol to below 10 % of the final volume to ensure complete enzyme activity.

A standard form was drawn up and enzyme digestion conditions were filled in along with the identification of samples and any informative notes. (Figures 7. and 8.)

FIGURE 7. STANDARD FORM 1.

Gel. Electrophoresis Date:- Agarose:-

% Gel:- Buffer:- Gel volume:-

Comb size:- Sample Vol:- Eth. Bromide:-

gel. photograph

DNA MARKERS

λ HindIII Size	Cms	1 Kb ladder Size	Cms	λ EcoRI Size	Cms
23.1		12.2		21.2	
		11.2			
9.4		10.2		7.4	
		9.2			
6.6		8.1		5.8	
		7.1			
4.4		6.1		5.6	
		5.1			
2.3		4.1		4.9	
		3.1			
2.0		2.0		3.5	
		1.6			
0.56		1.0			
		0.5			

Conditions for running gel.

Voltage:- Current:- Time:-

Depurination:-
Denaturing:- Neutralising:-

Conditions for blotting

Labelling Probe:-

Prehybridisation

Hybridisation

Comments.

FIGURE 8. STANDARD FORM 2.

Enzyme:-		Conc^{n}.:-			Date :-				
Supplier:-					Samples:-				
Sample No.	Sample I.D.	µl DNA	µl React. Buffer	µl Enzyme	µl Water			Total Volume	Any Remarks.
1.									
2.									
3.									
4.									
5.									
6.									
7.									
8.									
9.									
10.									
11.									
12.									
13.									
14.									
15									

Digest Conditions

Agarose gel electrophoresis.

The standard method for separating and analysing DNA fragments is by electrophoresing the nucleic acid mixture in agarose gels. A voltage applied at the ends of the gel sets up an electric field, and DNA molecules exposed to this field will migrate towards the positive electrode, the anode, due to the negatively charged phosphates along the DNA backbone. The strength of this field is defined from the length of the gel and the potential difference at the ends in units of Volts / cm. The migration rate depends on :

1. The molecular size.

Duplex linear DNA travels through agarose at a rate that is inversely proportional to the $\log_{10}$ of the molecular weight.(Helling. 1974)[38]

2. The agarose concentration.

DNA fragments of known sizes migrate at different rates through different concentrations of agarose. Therefore it is possible to resolve a wide size range of DNA fragments by changing the percentage of agarose used.

Amount of agarose in gel (%)	Range of separation Kb
0.3	60 - 5.0
0.6	20 - 1.0
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

3. Conformation of the DNA.

DNA can exist in three conformational forms and the same molecular weight will migrate at a different rate depending on the conditions used in electrophoresis. (Thorne.1967)[39]

Form I - closed circular DNA

Form II - nicked circular DNA

Form III - linear DNA

4. Applied voltage.

At low voltages DNA fragments migrate through the agarose gel at a rate proportional to the voltage applied. However with increasing voltages, larger fragments of DNA travel proportionally faster than the smaller fragments and the effective range of separation diminishes.

For most of the analysis carried out a 1% agarose gel was used but in the case of larger DNA fragment sizes above 12 kb, 0.8% or 0.5% gels were used.

Molecular biology grade agarose from Pharmacia was used which was DNase and RNase free.

Two buffer systems were used in the agarose gel electrophoresis. Initially 1 x TBE was used, due to its high buffering capability on overnight electrophoresis runs. However it was found that the DNA fragments were held back in the agarose on Southern blotting and made transfer of the DNA

less efficient. It was used for higher voltage gels run over shorter periods of 1 or 2 hours.

1 x TAE was the selected buffer and although having a lower buffering capacity, DNA transfer was greatly improved. For overnight runs a circulating buffer system was used to stop 'ionic exhaustion' over extended periods of electrophoresis, resulting in the anode becoming alkaline and the cathode becoming acidic.

The agarose was weighed out into a conical flask and the appropriate amount of 1 x TAE buffer added and melted by boiling in a microwave oven for several minutes until all the particles of agarose were completely dissolved. The flask was covered and left to cool to about 55^{o}C, just comfortable to be held in the hand, and ethidium bromide added at a concentration of 0.5 μg / ml.

A plastic gel casting tray that was translucent to UV light was sealed at the two open ends with sealing tape, autoclave tape was used as it was still adhesive when wetted. The tray was set up on a levelling table and a suitable gel comb fixed into the tray to leave a 3 mm gap between the bottom of the comb and the base of the tray. The size of the comb was chosen depending on the amount of DNA sample to be loaded and the thickness of the gel.

The usual size comb, for up to 50 μl samples, was 6 mm x 1 mm in a gel 1 cm deep. The number of wells depended on the gel size. A 20 cm x 24 cm gel used 1 comb with 20 wells, or 2 combs in parallel, making 40 wells, but this limited the

migration distance by half. This size gel was made with 300 ml of agarose solution.

The cooled agarose was poured into the tray taking care not to trap any air bubbles on the comb or on the surface of the gel. These were removed with a pasteur pipette. The gel was left to set for 30 minutes before the comb and the autoclave tape were carefully removed.

Low percentage gels were cooled at 4°C to give further rigidity and prevented the wells from tearing on removal of the comb.

The solidified gel, in the tray, was placed in the electrophoresis tank and the same buffer as the gel was made up in, was used as the running buffer. This was poured into the tank until the gel was submerged to a depth of 1 mm, and no air pockets were trapped in the wells.

10 x loading buffer was added to the restricted DNA samples at one - tenth final concentration. e.g. 5 µl of loading buffer in 50 µl of DNA sample. The loading buffer made the samples sufficiently dense to sink to the bottom of the wells and contained a tracking dye that ran as a blue coloured front, approximating to the migration of 500 base DNA, monitoring the progress of the separation.

The samples were loaded into the wells using a micro-pipettor and care was taken not to overfill the wells and cause sample mixing. DNA size markers were also loaded , to ascertain the band sizes of the polymorphisms.

The lid and the integral leads were connected so that the DNA

samples ran towards the positive electrode (anode), and the voltage set, depending on running time, normally 50 volts for an overnight run on a 24cm length gel.

The circulating buffer system pump, used for running TAE buffer gels, was turned on after the samples had electrophoresed out of the wells and into the gel.

After electrophoresis, the gel was lifted in its casting tray and positioned over a medium wave UV transilluminator. The ethidium bromide, bound to the DNA, fluoresced under the influence of the UV source, and made the DNA fragments visible.

A ruler was placed alongside the gel and a Polaroid photograph taken.

FIGURE 9.

POLAROID PHOTOGRAPH OF DNA SAMPLES RESTRICTED WITH BGL I.

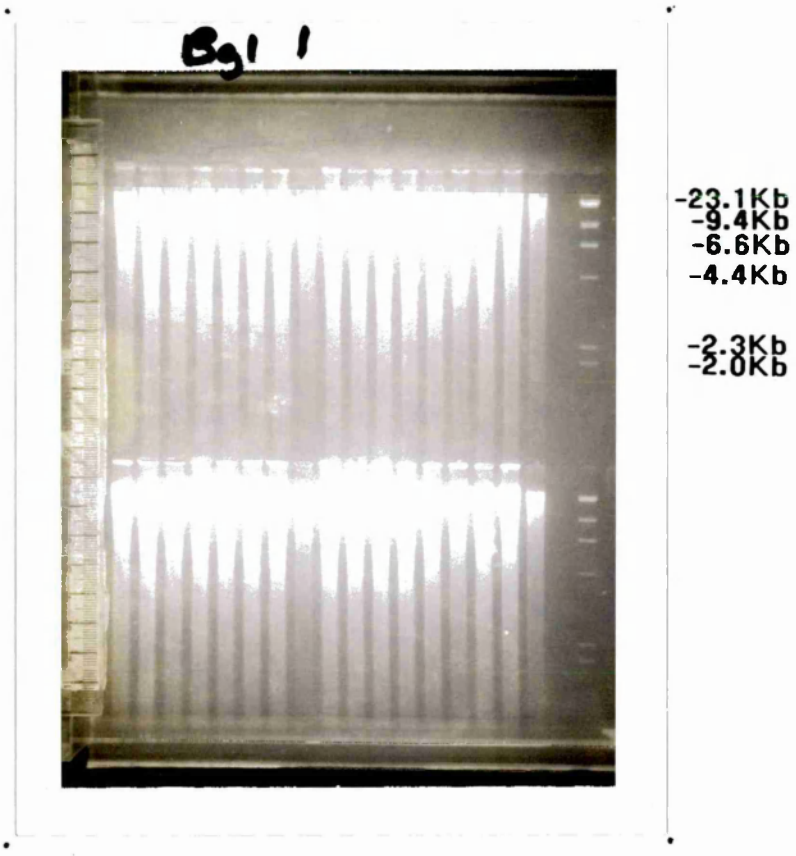


FIGURE 10.

PHOTOGRAPHING GELS POSITIONED ON THE TRANSILLUMINATOR.

Gel denaturation and neutralisation.

The corner of the gel, nearest the well that was first loaded, was cut off to act as an orientation mark.

The gel was placed into a tray containing enough denaturing solution to cover the gel and slowly rocked for a minimum of 1 hour. For gels where the size of the DNA fragments of interest were larger than 10 kb the gel was first put into depurination solution for half an hour.

Depurinated sites on the DNA were cleaved during the alkali treatment and made smaller DNA fragments transfer more efficiently.

Denaturing solution was removed from around the gel using a 10 ml pipette attached to a water vacuum pump, and neutralising solution poured on to just cover the gel and rocked gently for 1 hour.

It was found that with transfer onto nylon membranes it was not necessary to neutralise the gel first, so the neutralising step was omitted and the gel was rinsed in de-ionised water.

Solutions used in agarose gel electrophoresis.

TAE (Tris - acetate)

For 1 litre of 10 x working solution

48.4 g Tris base

20 ml 0.5 M EDTA pH 8.0

approx 14 ml glacial acetic acid

to make final solution pH 8.0

made up in distilled water

Working solution concentration

0.04 M Tris - acetate

0.002 M EDTA

TBE (Tris - borate)

For 1 litre of 10 x working solution

108 g Tris base

55 g Boric acid

20 ml 0.5 M EDTA pH 8.0

made up in distilled water

Working solution concentration

0.089 M Tris

0.089 M boric acid

0.002 M EDTA

Ethidium bromide solution

For 100 ml of 1000 x working solution

50 mg ethidium bromide

100 ml distilled water

stored in foil covered amber bottle

Working concentration

0.5 µg / ml

Loading buffer

For 50 ml of 10 x working solution

0.4 g bromophenol blue

50 ml glycerol

stored at 4°C

Depurination solution

For 1 litre of 0.2 M HCl

17.2 ml concentrated Hydrochloric acid

added carefully to 982.8 ml distilled water

Denaturing solution

For 1 litre of working solution

20.0 g NaOH

87.46 g NaCl

made up to 1 litre with distilled water

Working solution concentration

0.5 M NaOH

1.5 M NaCl

Neutralising solution

For 1 litre of working solution

65.52 g Tris

87.46 g NaCl

made up with distilled water and to pH 7.5 with a few drops of concentrated hydrochloric acid

Working solution concentration

0.5 M Tris

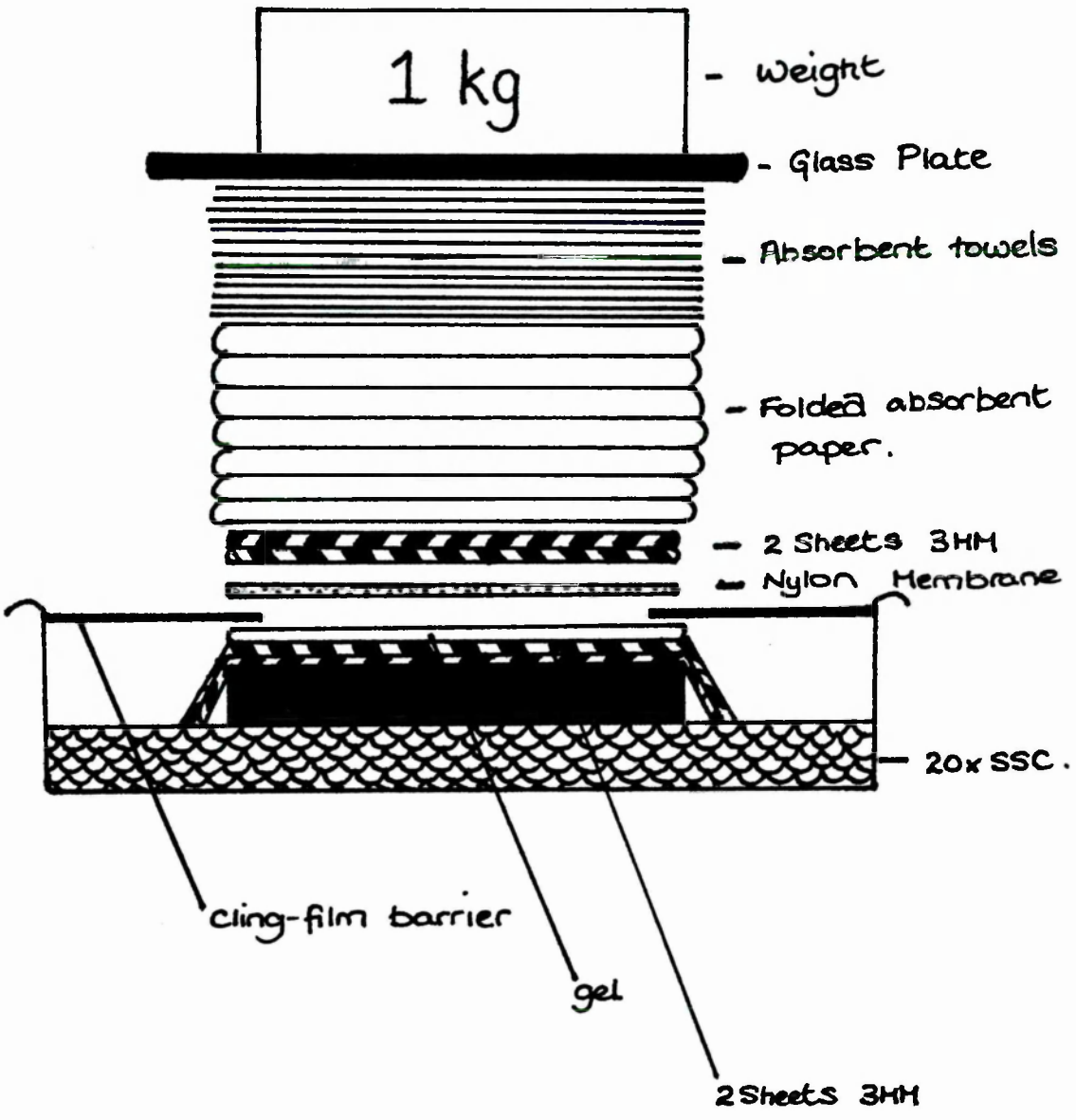
1.5 M NaCl

Southern blotting.

Southern blotting, the capillary transfer and immobilisation of DNA fragments onto various support filters or membranes was first described by E.M.Southern (1975)[40]. He used cellulose nitrate filters but it was found that nylon membrane filters (Hybond N manufactured by Amersham) were easier to handle and did not break up on hybridisation.

The Southern blotting apparatus (see figure 11.) consisted of a plastic tray inverted in a larger tank with two pieces of Whatman 3MM chromatography paper cut to cover the tray and overlap to form wicks down into the tank. Enough 20 x SSC was poured over the tray and into the tank to completely soak the paper and immerse the wicks, and a tube used to roll over the wet surface to push out any air pockets. The agarose gel was carefully slid onto the covered tray and rolled over again with the tube to ensure a good contact with the surface. Hybond N nylon filter membrane was cut to the same size as the gel. Gloves were used to handle the membrane to prevent transfer of contaminants off the hands. It was laid onto the gel making sure it was flat and the tube rolled across the surface. With this type of membrane it was not necessary to prewet it. To prevent the SSC buffer from seeping up around the sides of the gel, a piece of plastic film was stretched along each edge of the gel and membrane, to form a barrier. Two pieces of 3MM chromatography paper were wetted with distilled water and placed over the membrane, followed by an inch depth of folded absorbent paper and a pile of paper towels, topped with a flat glass plate with a 1 kg weight.

FIGURE 11. SOUTHERN BLOTTING APPARATUS



The transfer by blotting was normally carried out overnight but similar results could be obtained by blotting for a minimum of 4 hours with a regular change of paper towels.
After blotting the towels and paper were removed carefully so as not to disturb the membrane and the gel wells located and marked with a ball-point pen. The corner of the membrane was cut to that of the gel to indicate orientation and the membrane was lifted off and placed, gel-touching side down, into a tray containing 6 x SSC and shaken gently for 30 seconds. This was to wash off any adhering agarose which would bind the probe and add to the non-specific background signal.
The membrane was laid onto 3MM paper and air dried for 15 minutes before being sandwiched between two sheets of 3MM and baked in an oven at 80^{o}C for 2 hours, to immobilise the DNA.
The baked membrane was covered in plastic Saran wrap and stored flat at room temperature until the hybridisation step.
Many techniques have been used to speed up the transfer and fixation of the DNA out of agarose gels.
Some of these were tried out in earlier experiments but none of them gave as good as results as the method described above.
Other methods employed:-

<u>Transfer using a vacuum blotter.</u>

The denatured gel was positioned on a vacuum blotter consisting of a nylon membrane resting on a fine mesh

platform. The vacuum was applied to the base of the apparatus and a small amount of buffer applied to the top of the gel which was gradually drawn through, carrying the DNA out of the gel and onto the membrane.

This method did not work as the gel was 1.2 cm thick and the vacuum necessary to draw the buffer through had to be set above the maximum working setting which made the gel collapse and hence trap the DNA. Thinner gels were tried but because the well volume was less, the amount of restricted DNA sample was inadequate to give a probe signal on hybridisation.

Transfer using an electroblotter.

The denatured gel was equilibrated in transfer buffer (0.025 M sodium phosphate pH 6.5) and the gel sandwiched in a plastic cassette with the membrane held next to the gel on the anodal side of the electroblotting apparatus. The tank was filled with the transfer buffer and the electrodes attached either side. A current of between 0.6 - 2 amps was the suggested working current for 1 - 2 hours.

Transfer at the higher current resulted in the buffer overheating and the gel melting. At the lower current, 0.6 amps, transfer was tried over a 2 hour period and overnight with cooling. This was accomplished by positioning the electroblotter tank inside a 4^{o}C incubator. The results showed poor transfer of DNA out of the gels revealed by post-electroblotting staining of the gel in ethidium bromide.

UV fixation of DNA onto nylon membrane

After Southern blotting with 20 x SSC, the nylon membrane was washed in 2 x SSC and left to air dry for 10 minutes. It was covered in plastic Saran wrap and placed on top of the transilluminator, DNA side down, and exposed to UV light for different lengths of time, ranging from 2 minutes up to 10 minutes. Results after autoradiography showed no distinct hybridisation bands which was probably due to insufficient cross-linking of the DNA onto the nylon membrane or the DNA having been nicked by stray short-wave UV light.

The UV crosslinking method of fixing DNA onto nylon membrane can only be used when the wavelength of the transilluminator is regularly calibrated. The recommended wavelength of 312 nm is critical, and test strips for exposure time and distance have to be verified regularly as UV tubes in the transilluminator become less intense with age.

This method was not therefore used .

Prehybridisation and hybridisation.

The hybridisation reaction, or the base pairing of homologous sequences of single stranded DNA, is complex and depends on many parameters. The formation of nucleic acid hybrids is a reversible process and optimal conditions are necessary to form perfect stab le DNA - DNA hybrids. The melting temperature (T_m) is defined as the temperature when half the duplex molecules have dissociated into their single strand components. This effects the conditions at which hybridisation and washing are carried out. The salt concentration (monovalent ions) effects T_m and a ten-fold molar increase in salt concentration raises T_m by 16°C.

eg. The T_m of mammalian DNA is approximately 85°C using 0.18 M Na^+ and rises to approximately 97°C using 1M Na^+.

The base concentration of the DNA will effect T_m. Hydrogen bonding between the two base pairings is different, adenine and thymine having a double bond and guanine and cytosine having a triple bond. The G-C base pairing will therefore be more stab le and the higher the proportion of these bases in the DNA - DNA hybrid will result in a higher T_m. The rate at which the duplexes form, depends upon the concentration of probe and target sequences, and the rate of hybridisation is inversely proportional to the sequence complexity and directly proportional to the nucleic acid concentration.

The complexity of Genomic Southern blots with single-copy genes, and the long cDNA probe length, results in having to use long hybridisation times, normally overnight, to enable effective matching to occur.

Prehybridisation and hybridisation solutions.

The same solutions were used for prehybridisation and hybridisation.

Prehybridisation and hybridisation buffer.

For 100 ml.

20.0 ml 20 x SSC

2.0 ml 100 x Denhardts solution

5.0 ml 10% SDS

0.1 ml 1 M EDTA

10.0 ml 1 M Phosphate buffer pH 7.0

1.0 ml denatured herring sperm DNA (10mg/ml)

6.0 g polyethylene glycol compound M.W. 15,000-20,000

41.9 ml sterile deionised water

100 x Denhardts solution

For 500 ml

10 g Ficoll 400 (Pharmacia)

10 g polyvinylpyrrolidone

10 g bovine serum albumin, fraction V (Sigma)

made up to 500 ml with sterile deionised water

filtered and stored in 10 ml aliquots at -20°C

1 M Phosphate buffer pH 7.0

61 ml 1 M di-sodium hydrogen orthophosphate

39 ml 1 M sodium di-hydrogen orthophosphate

The hybridisation solution was stored at -20°C .

Phosphate hybridising buffer.

0.5 M Phosphate buffer pH 7.0

7% SDS

Made by adding equal quantities of 1 M phosphate buffer pH 7.0 to a 14% solution of SDS, stored at room temperature in 50 ml aliquots and mixed well before use.

1 M Phosphate Buffer (pH 7.0)

Mix 100 ml of 1 M Na_2HPO_4 (358.14 g / 1.) with approx. 60 ml of 1 M $NaH_2PO_4.2H_2O$ (156.01 g / 1.) to make pH 7.0.

Results obtained when using the second hybridisation solution were comparable to those obtained from the first.

Hybridisation in polythene bags.

Polythene bags were made from clear heavy duty grade polythene tubing cut to size and sealed with a foot-operated bag sealer. The size of the bag was made to fit the nylon membrane so that the minimum of hybridisation solution was used, increasing the probe concentration.

The membrane was placed flat inside the bag and sealed leaving a small opening. 20 ml of prehybridisation solution was pipetted in, and air bubbles removed by squeezing the bag until a small amount of the fluid was expelled, and the bag fully sealed.

The bag was placed into a plastic box half filled with water and covered with a polystyrene float, to keep the membrane flat. The box was put into a 65^{o}C shaking waterbath for a minimum of 1 hour.

Constant agitation ensured that the membrane was evenly wetted with the solution and that no air bubbles adhered to it. The bag was removed, the corner cut, and the prehybridisation solution squeezed out.

Hybridisation solution, 100 µl / cm^2 of membrane, was pipetted into a 30 ml Universal tube and the denatured radioactive probe added and mixed. This solution was transferred into the bag using a pasteur pipette. Air bubbles were removed carefully and the bag sealed and hybridisation carried out overnight in similar conditions as for prehybridisation.

Hybridisation in tubes.

An alternative to using polythene bags for hybridisation is to use tubes rotated inside a thermostatted oven.

The nylon membrane was rolled up and inserted into a 100 ml plastic tube. 10 ml of prehybridisation solution was pipetted into the tube and a silicone bung inserted, with a syringe needle inset, to stop pressure build-up. The tube was inverted laterally into the rotating arm of a hybridising oven set at 65°C, the door closed and the rotator motor switched on.

Prehybridisation was carried out over a minimum period of 10 minutes.

The denatured probe was added directly into the prehybridisation solution in the tube and hybridisation continued overnight.

The advantage of using the 'tube in the oven' method over the bag method, was that the hybridisation solution was in contact with the filter continuously with the constant rotation, whereas the bags tended to float unevenly and produce patchy hybridisation. It was also difficult to expel all the air bubbles without some spillage of radioactive solution.

Removal of non-specific binding.

The membranes were carefully removed from the bags or tubes and placed flat into a sandwich box containing enough solution A to cover them. The box was covered and placed on a shaking platform at room temperature for 5 minutes. The solution was replaced with fresh solution A and washed again, with agitation, for a further 5 minutes.

The membranes were removed and placed flat on top of a plastic sheet and monitored with a Geiger-Müller tube type mini-monitor. (Mini-instruments Ltd.)

A uniform count rate across the whole of the membrane showed that the washing was not stringent enough.

The membrane was then washed successively more stringently in solutions B and C and the temperature of the solutions raised until distinct radioactive areas with non radioactive areas were monitored.

The membrane was wrapped in plastic Saran wrap and autoradiographed. It was essential that the membrane was not allowed to dry out.

Using nylon membranes allowed the removal of one radioactive probe and reprobing with another.

Membrane washing solutions

(A) : 2 x SSC , 0.1% SDS

(B) : 0.2 x SSC, 0.1% SDS

(C) : 0.1 x SSC, 0.1% SDS

Removing radioactive probe from nylon membranes.

Two methods were used for removing the radioactive probe from the membranes.

The membrane was submerged in a boiling solution of 0.1% SDS and left to cool to room temperature.

This method was found not to be stringent enough and on autoradiography of the stripped membrane, probe signal was still detectable.

A more stringent method was employed, by incubating the membrane at 45°C for 30 minutes in 0.4 M NaOH and transferring the membrane into:

0.1 x SSC

0.1% SDS

0.2 M Tris-HCl pH 7.5

and incubating at 45°C for 30 minutes.

This method removed most of the signal but on reprobing the non-specific background signal was high and resulted in unclear band patterns.

Therefore, for most blots, it was better to repeat the Southern analysis from the beginning.

Autoradiography.

Autoradiography is used to visualise and quantitate, on X-ray film, radioactive molecules hybridised onto the membranes. To enhance the signal, solid state scintillation, intensifying screens, are employed to convert the radioactive energy to visible light. Beta particles activate the silver bromide crystals on a film emulsion and are reduced through development to silver metal (grain). To slow the reversal of the activated form back to the stab le form the exposure is conducted at low temperature. Intensifying screens, made from calcium tungstate, are used to enhance the radioactive signal. Emissions from strong beta particles pass completely through the film but can be absorbed by the screens, which fluoresce and return them back to the film as multiple photons of light.

The covered membranes were labelled with identification marks written in radioactive ink and loaded into an autoradiography cassette in the following order:

1. The back intensifying screen.
2. The X-ray film.
3. The tube intensifying screen.
4. The membrane face down.

The cassette was closed and labelled and placed into a -70^{o}C freezer overnight, or for membranes with a weaker signal, up to 1 week.

The cassette was removed and left to equilibrate to room

temperature before removing the exposed film. This prevented blackening of the film due to static caused by the interaction of ice crystals on plastic.

The X- ray film used was either X-omat AR(Kodak) or Hyperfilm-MP (Amersham) and it was developed under red safelight as follows:

5 minutes in developer with slight agitation.

10 seconds in stop-bath.

2 minutes in fixer

10 minutes in constantly changing water.

All solutions were equilibrated to room temperature (23°C) and all procedures were performed in open trays.

Developer

LX 24 (Kodak)

Dilution : 8ml concentrate plus 450 ml water

Stop bath

Indicator stopbath (Kodak)

Dilution : 16ml concentrate made up to 1 litre with water.

Fixer

FX 40 (Kodak)

Dilution : 1 part concentrate to 3 parts water

The film was dried and the autoradiograph identification marks were superimposed onto those on the membrane, the sample well tracks were marked and the polymorphic bands measured and recorded.

Physiological measurements

In the second study group, plasma ANP and aldosterone measurements and urinary sodium levels were performed by Dr. Alberto Smith using radioimmunoassay and atomic absoption spectrophotometry techniques already employed in the department of Child Health.

In the blood pressure study group, plasma renin levels were measured by Dr. Brenda Leckie from the MRC Blood Pressure Unit, Glasgow, using an antibody trapping technique.(Millar. 1980)[41]

Statistical methods used for analysis of the data.

Chi-squared analysis.

The Null Hypothesis states that any difference is due entirely to chance and the alternative to this, is that any difference is significantly more than could be reasonably expected by chance.

The chi-square test measures the size of discrepancy between observed and expected results and for each value gives a probability of occurrence. The larger this discrepancy value or chi-square value, the less probable the occurrence, and given the number of degrees of freedom, this probability can be established from a chi-square distribution table.

For testing three genotypes, there are two degrees of freedom and from the table, the probability of values above 5.991 are sufficiently rare, significance level 0.05, that the null hypothesis can be rejected and therefore the difference between the observed and expected genotype arises from some factor other than chance.

Chi ^{2} formula:

$$\chi^2 = \frac{(O - E)^2}{E}$$

where O is the observed number and E is the expected number.

When the numbers of expected and observed are small (less than 30) the chi-square determination is made more accurate by using Yates correction.

The formula then becomes:

$$\chi^2 = \frac{\{ (O - E) - 0.5 \}^2}{E}$$

Calculation of expected values from a contingency table of observed results.

$$E = \frac{\text{row total x column total}}{\text{grand total}}$$

Where E = expected results.

Example: Testing the frequency of allelic polymorphisms T, TB, and B in hypertensives (H) and controls (C)

Observed

	H	C	Total
T	17	44	61
TB	12	46	58
B	6	6	12
Total	35	96	131

Expected

	H	C
T	16.2	44.7
TB	15.5	42.5
B	3.2	8.8

Substituting these figures into the chi-squared formula with Yates correction.

$$\frac{\{(17-16.2)-0.5\}^2}{16.2} + \frac{\{(44-44.7)-0.5\}^2}{44.7} + \frac{\{(12-15.5)-0.5\}^2}{15.5}$$

$$+ \frac{\{(46-42.5)-0.5\}^2}{42.5} + \frac{\{(6-3.2)-0.5\}^2}{3.2} + \frac{\{(6-8.8)-0.5\}^2}{8.8}$$

$$= 0.006 + 0.054 + 1.03 + 0.21 + 1.65 + 1.24 = 4.19$$

Chi-square value = 4.19 with 2 degrees of freedom. $p > 0.05$. Accept the null hypothesis. The difference in genotype in hypertensives and controls may be caused by chance.

Frequency distribution and correlation coefficient.

In the second study group multivariable analysis was undertaken on the different genotypes and physiological measurements.

Frequency distributions were determined on all physiological values for each genotype to look for trends in high or low values associated with a particular genotype.

The product-moment correlation coefficient (r) was determined for each combination of pairs of physiological measurement and a probability value determined.

Log-linear modelling.

In the third study group more sophisticated statistics were used to show an association between three factors, blood pressure, ethnic group and genotype. This log-linear modelling (a computer based three dimensional table) was analysed by Mr. A. Charlett from the department of Medical statistics at Northwick Park Hospital, Harrow.

CHAPTER 3. RESULTS.

CHAPTER 3. RESULTS.

Section 1. Restriction fragment length sizes.

Restriction fragment length polymorphisms were determined for each gene probe, on the different study groups, using restriction enzymes previously reported as having polymorphic fragment lengths for the gene locus of interest. It was noted that the fragment sizes obtained, with reference to DNA markers run on the same gels were sometimes different from the reported sizes.

These differences were attributed to:

a) The migration rate of DNA changing when using different percentage agarose gels, and buffer systems. Bands that were sized on a 1% gel differed when sized on a 0.8% gel. This could also be a measuring problem, as lower percentage gels tended to squash down more readily, on Southern blotting, and the accurate marking of the gel loading wells, on the nylon membrane, proved more difficult.

b) That different amounts of DNA loaded onto the gels affected the migration rate. The amount of DNA in the markers was less than in the digests and it was found that samples with more DNA in, ran faster.

c) The accuracy of reading off a curved slope on a graph.

All bands were sized against at least two different marker sets.The following tables show the restriction fragment sizes as reported and those that were observed.

All the fragment sizes stated in the results section were those first reported in publication. The Kallikrein RFLP had not been published and therefore the observed size was used.

TABLE 3. REPORTED AND OBSERVED FRAGMENT SIZES USING h-PND.

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb
PND	Xho I	12	10	variable incomplete digest
	Bgl I	6.2	4.1	9.0 6.5 3.5 incomplete digest
Other enzymes showing non-polymorphic bands				
	Bgl II			6.5
	Hind III			9.0
	Msp I			3.0 1.8

TABLE 4. REPORTED AND OBSERVED FRAGMENT SIZES USING ANF.

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb	
ANF	Bgl I	6.2	4.1	6.2	4.1
	Bgl II	9.1	6.5	9.2	7.3
	Sca I	10	7.4,2.6	12.6	9.5, 3.1
Other enzymes showing non polymorphic bands					
	Hind III				9.0
	Nco I				1.5
	Taq I			multiple bands	
	Eco RI				^ 10.0

TABLE 5. REPORTED AND OBSERVED FRAGMENT SIZES USING 3'RENIN.

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb	
3'RENIN	Hind III	9.0	6.2	9.2	6.4
	Other enzymes analysed showing non polymorphic bands				
	Taq I			3.5	2.3
	Eco RI, Bgl I, Bgl II			no bands	

TABLE 6. REPORTED AND OBSERVED FRAGMENT SIZES USING 5'RENIN.

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb	
5'RENIN	Bgl I	9.0	5.0	10.0-9.0	5.5-5.2
		11.0	6.0		
	Bgl II	24.0	20.0	^ 28.0	23.0
		25.0	22.0		
	Taq I	11.0	9.8	11.0	9.5
		11.0	10.0		
	Other enzymes analysed showing non polymorphic bands				
	Hind III Sca I				
	Eco RI				

TABLE 7.REPORTED AND OBSERVED FRAGMENT SIZES USING KALLIKREIN

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb	
KALLIKREIN	ECO RI	<12	9.5	<12	9.5

Other enzymes analysed showing non polymorphic bands

Bgl I Bgl II Hae III Hpa I

Pvu II Sca I Sau 96I Xho I

Hind III Pst I Taq I

Rsa I

TABLE 8. REPORTED AND OBSERVED FRAGMENT SIZES USING GCR.

GENE PROBE	RESTRICTION ENZYME	REPORTED SIZE Kb		OBSERVED SIZE Kb	
GLUCOCORTICOID RECEPTOR	BCl I	4.5	2.3	3.5	2.1

Section 2. Study group 1. Hypertensives V Controls.

The difference in the number of samples tested in each group was due to inadequate amount of DNA sample for some of the analyses.

TABLE 9.

ANF probe on **Bgl I** digests.

Polymorphism T = 6.2Kb B = 4.1Kb

	6.2:6.2	6.2:4.1	4.1:4.1	Allele frequency
Hypertensives	25	11	3	0.78 / 0.22
Controls	23	7	0	0.88 / 0.12

Chi-square = 2.85

Degrees of freedom = 2 P = 0.2409

Chi-square test not valid. Cells with expected value < 5 = 2

TABLE 10.

ANF probe on **Bgl II** digests.

Polymorphism T = 9.1Kb B = 6.5Kb

	9.1:9.1	9.1:6.5	6.5:6.5	Allele frequency
Hypertensives	0	0	33	0 / 1.0
Controls	0	2	52	0.02 / 0.98

Chi-square = 1.25

Degrees of freedom = 2 P = 0.2634

Chi-square test not valid.Cells with expected values < 5 = 4

TABLE 11.

ANF probe on Sca I digests.

Polymorphism T = 10Kb B = 7.4Kb

	10:10	10:7.4	7.4:7.4	Allele frequency
Hypertensives	2	4	27	0.12 / 0.88
Controls	0	10	26	0.14 / 0.86

Chi-square = 4.47

Degrees of freedom = 2 P = 0.1086

Chi-square test not valid. Cells with expected value < 5 = 2

TABLE 12.

3' Renin probe on Hind III digests.

Polymorphism T = 9.0Kb B = 6.2Kb

	9.0:9.0	9.0:6.2	6.2:6.2	Allele frequency
Hypertensives	17	12	6	0.66 / 0.34
Controls	44	46	6	0.70 / 0.30

Chi-square = 4.44

Degrees of freedom = 2 P = 0.1086

TABLE 13.

5' Renin probe on Taq I digests

Polymorphism T = 11.0Kb B = 9.8Kb

	11.0:11.0	11.0:9.8	9.8:9.8	Allele frequency
Hypertensives	1	7	27	0.13 / 0.87
Controls	1	11	41	0.12 / 0.88

Chi-square = 0.09

Degrees of freedom = 2 P = 0.9544

Chi-square test not valid. Cells with expected vale < 5 = 2

TABLE 14.

5' Renin probe on Bgl I digests.

Polymorphism T = 9.0Kb B = 5.0Kb

	9.0:9.0	9.0:5.0	5.0:5.0	Allele frequency
Hypertensives	11	14	13	0.47 / 0.53
Controls	33	49	5	0.66 / 0.34

Chi-square = 17.48

Degrees of freedom = 2 P = 0.0002

FIGURE 12. PHOTOGRAPH OF AUTORADIOGRAPHS OF BGL I GENOMIC DIGESTS PROBED WITH ANF.

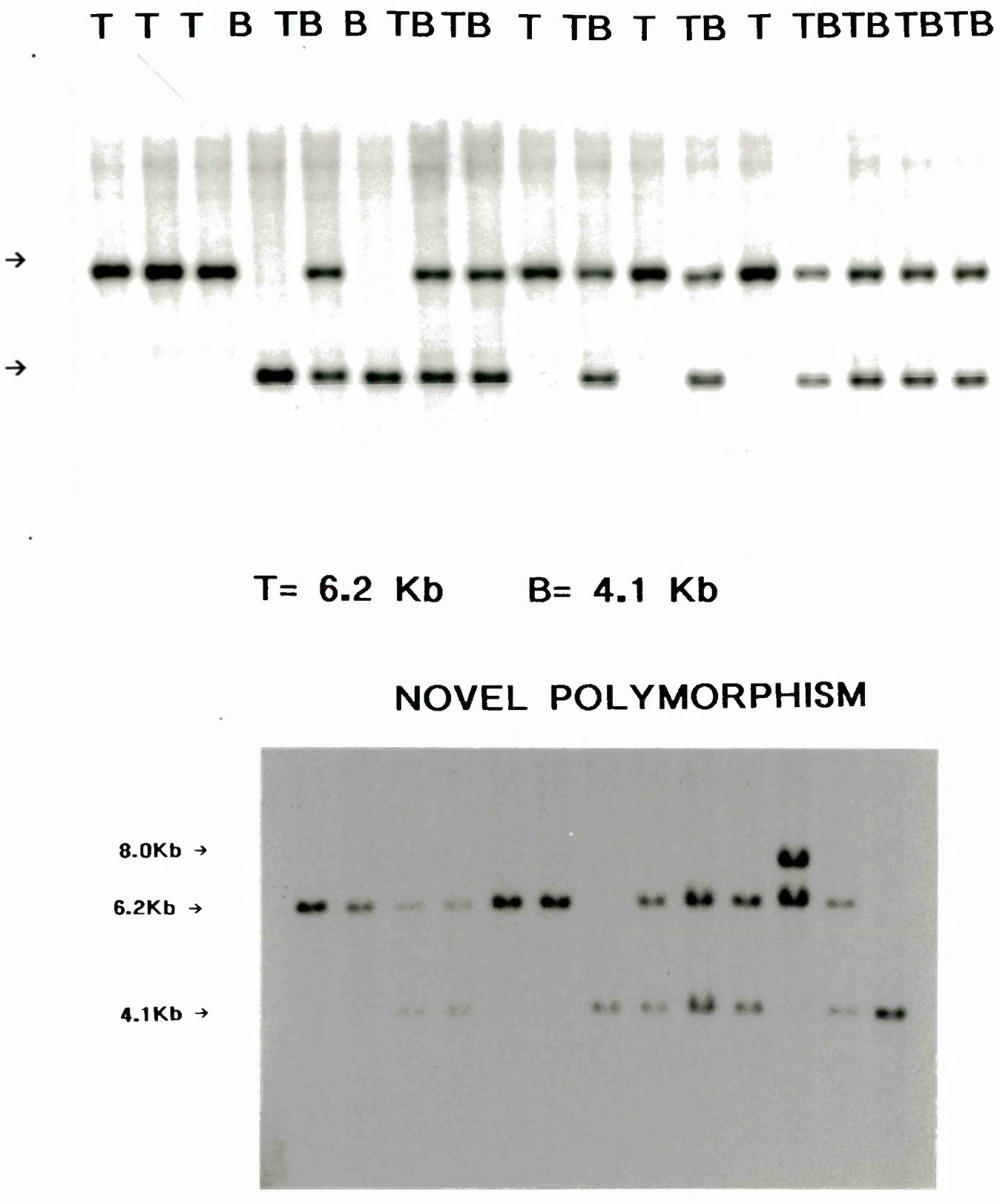
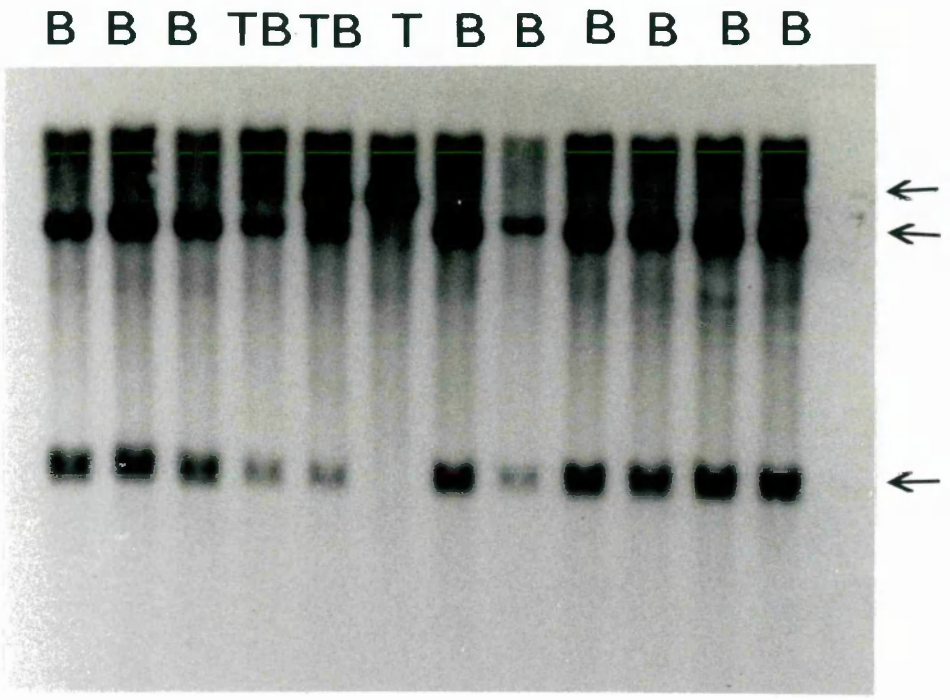


FIGURE 13. PHOTOGRAPH OF AUTORADIOGRAPH OF SCA I GENOMIC DIGESTS PROBED WITH ANF.



T= 10Kb B= 7.4Kb + 2.6Kb

FIGURE 14. PHOTOGRAPH OF AUTORADIOGRAPH OF BGL II GENOMIC DIGESTS PROBED WITH ANF.

T= 9.1Kb B= 6.5Kb

FIGURE 15. HISTOGRAMS SHOWING THE FREQUENCY OF RFLPS IN HYPERTENSIVE AND CONTROL GROUPS.

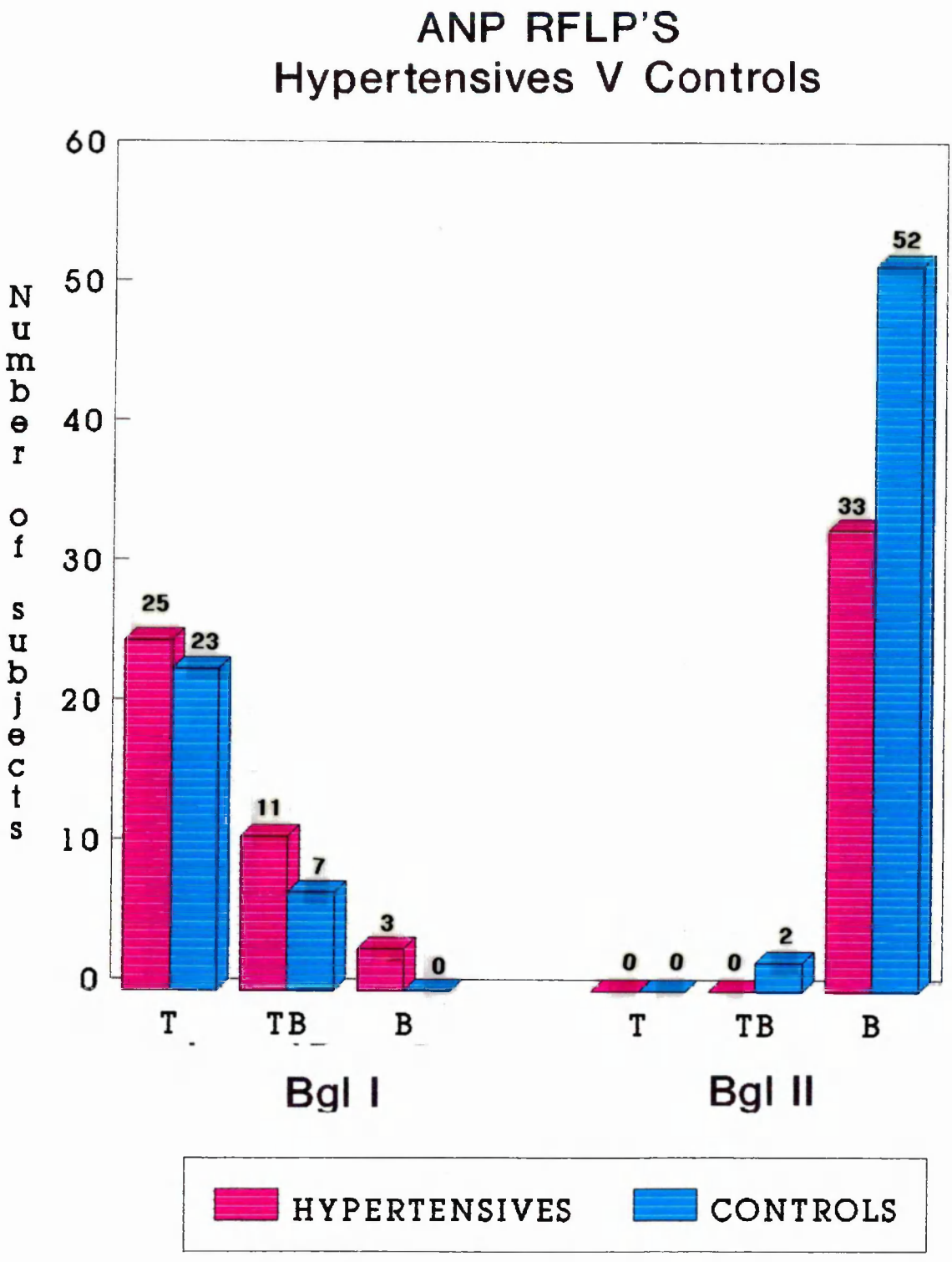
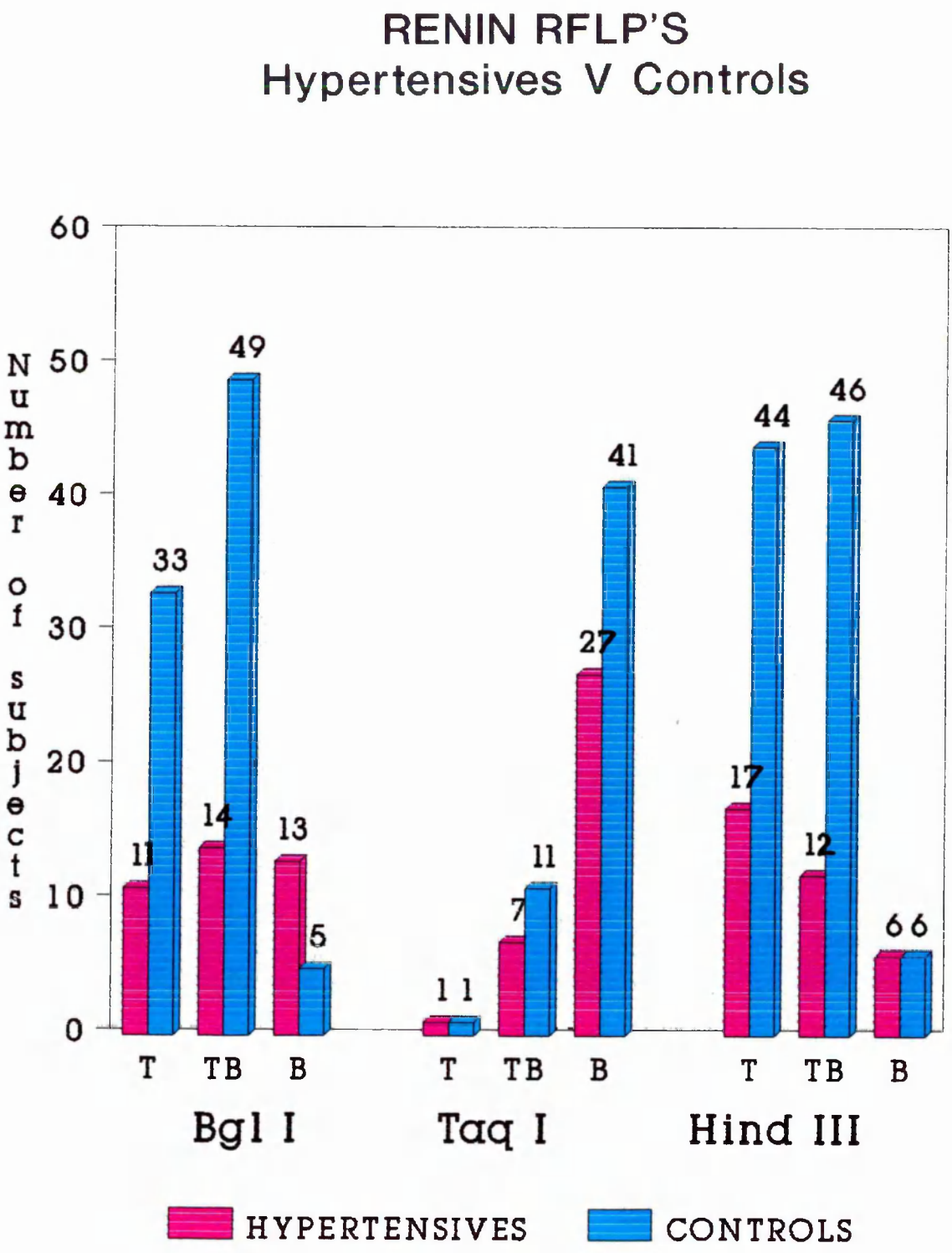


FIGURE 16. HISTOGRAMS SHOWING THE FREQUENCY OF RFLPS IN HYPERTENSIVE AND CONTROL GROUPS.



Section 3. Study group 2. Department controls.

TABLE 15. RFLPS USING THE RENIN PROBE.

	Bgl I	Bgl II	Hind III	Tag I
	T= 9 Kb	T= 24 Kb	T= 9 Kb	T= 11 Kb
	B= 5 Kb	B= 20 Kb	B= 6.2Kb	B= 9.8Kb
1.	T	B	T	B
2.	T	B	T	B
3.	TB	TB	TB	TB
4.	T	B	TB	TB
5.	T	B	B	B
6.	TB	B	T	B
7.	TB	B	TB	B
8.	T	B	TB	B
9.	TB	TB	B	TB
10.	TB	TB	T	TB
11.	TB	TB	B	TB
12.	T	B	TB	B
13.	TB	B	T	B
14.	T	B	TB	B
15.	TB	TB	TB	TB
16.	TB	B	T	B
17.	TB	TB	TB	TB
18.	TB	B	TB	B
19.	TB	B	T	B
20.	TB	B	TB	B

TABLE 16. RFLPS USING THE ANF PROBE.

	Bgl I	Sca I
	T= 6.2 Kb	T= 10 Kb
	B= 4.1 Kb	B= 7.4 Kb
1.	T	TB
2.	T	-
3.	T	TB
4.	T	TB
5.	TB	B
6.	T	B
7.	T	TB
8.	TB	TB
9.	TB	TB
10.	TB	B
11.	T	TB
12	T	TB
13.	TB	TB
14.	T	TB
15.	T	TB
16.	TB	TB
17.	T	TB
18.	T	TB
19.	T	TB
20.	TB	B

TABLE 17.

Department controls
Blood Pressure (mm Hg.)

Subject	Systolic BP	Diastolic BP	MAP
1	110	70	83.3
2	130	90	103.3
3	110	70	83.3
4	135	95	108.3
5	110	70	83.3
6	105	70	81.7
7	105	80	88.3
8	130	75	93.3
9	130	90	103.3
10	130	85	100.0
11	130	85	100.0
12	100	70	80.0
13	100	70	80.0
14	100	75	83.3
15	115	65	81.7
16	115	80	91.7
17	100	75	83.3
18	110	80	90.0
19	120	80	93.3
20	110	70	83.3

MEAN ARTERIAL PRESSURE =

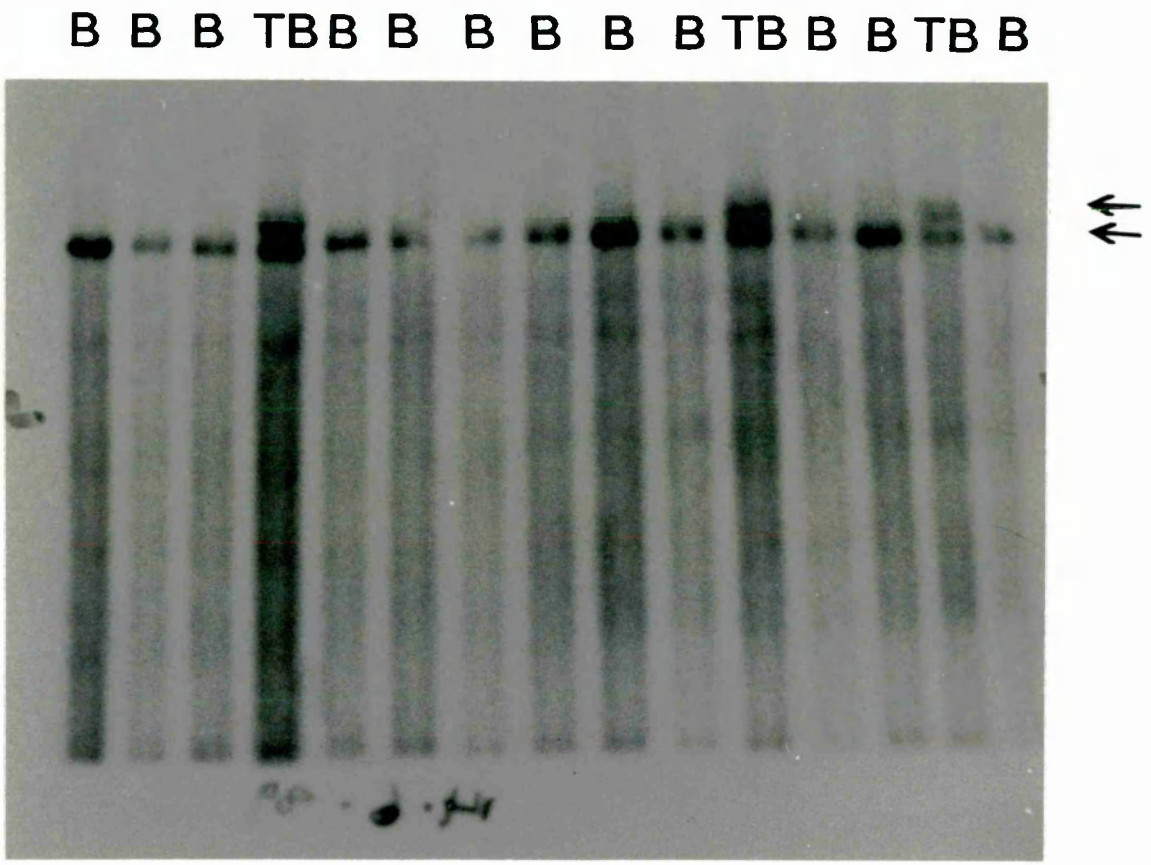
$$\frac{(2X\ \text{DIASTOLIC}) + \text{SYSTOLIC}}{3}$$

TABLE 18.

Department controls
Physiological measurements

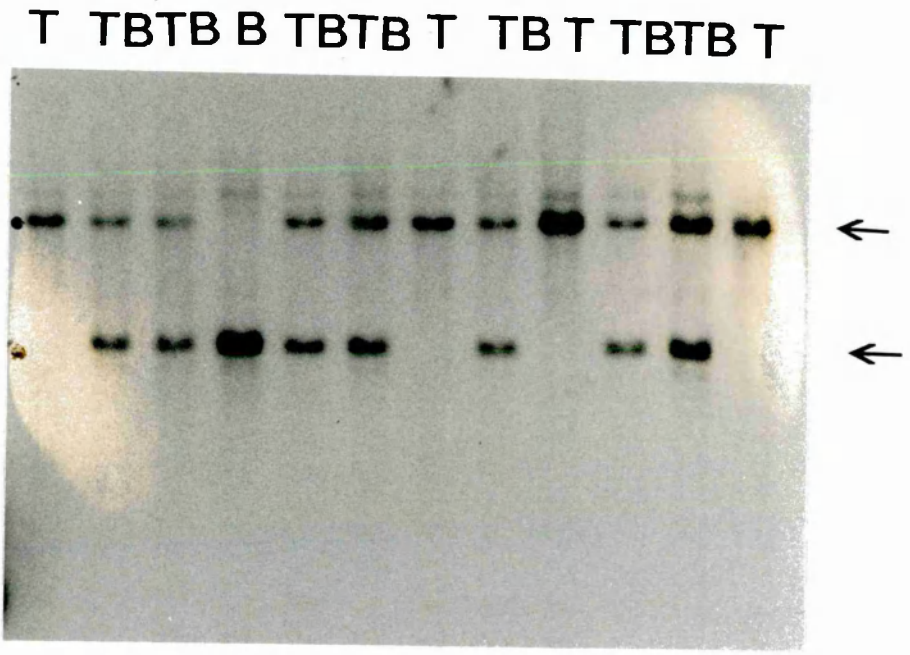
	Aldosterone pg/ml		ANP pg/ml		Sodium mmol/l
	T=0	T=30	T=0	T=30	
1.	117	104	13.9	5.4	44
2.	97	142	16.0	10.7	218
3.	171	195	5.4	5.7	43
4.	151	141	16.0	7.2	53
5.	135	132	0.8	1.3	89
6.	100	117	0.7	13.6	69
7.	173	158	1.5	1.1	127
8.	52	118	0.9	5.1	170
9.	118	126	14.7	12.6	98
10.	66	115	2.4	5.0	160
11.	112	103	4.8	21.0	43
12.	118	118	9.1	11.2	60
13.	246	160	13.7	25.1	44
14.	134	154	17.5	10.8	68
15.	157	165	12.0	12.1	84
16.	110	178	1.1	1.1	116
17.	111	85	3.1	3.9	174
18.	125	137	1.0	1.0	83
19.	106	107	29.4	18.3	95
20.	135	145	15.1	10.2	59

FIGURE 17. PHOTOGRAPH OF AUTORADIOGRAPH OF TAO I GENOMIC DIGESTS PROBED WITH 5'RENIN.



T= 11.0Kb B= 9.8Kb

FIGURE 18. PHOTOGRAPH OF AUTORADIOGRAPH OF BGL I GENOMIC DIGESTS PROBED WITH 5'RENIN.



T= 9.0Kb B= 5.0Kb

FIGURE 19.

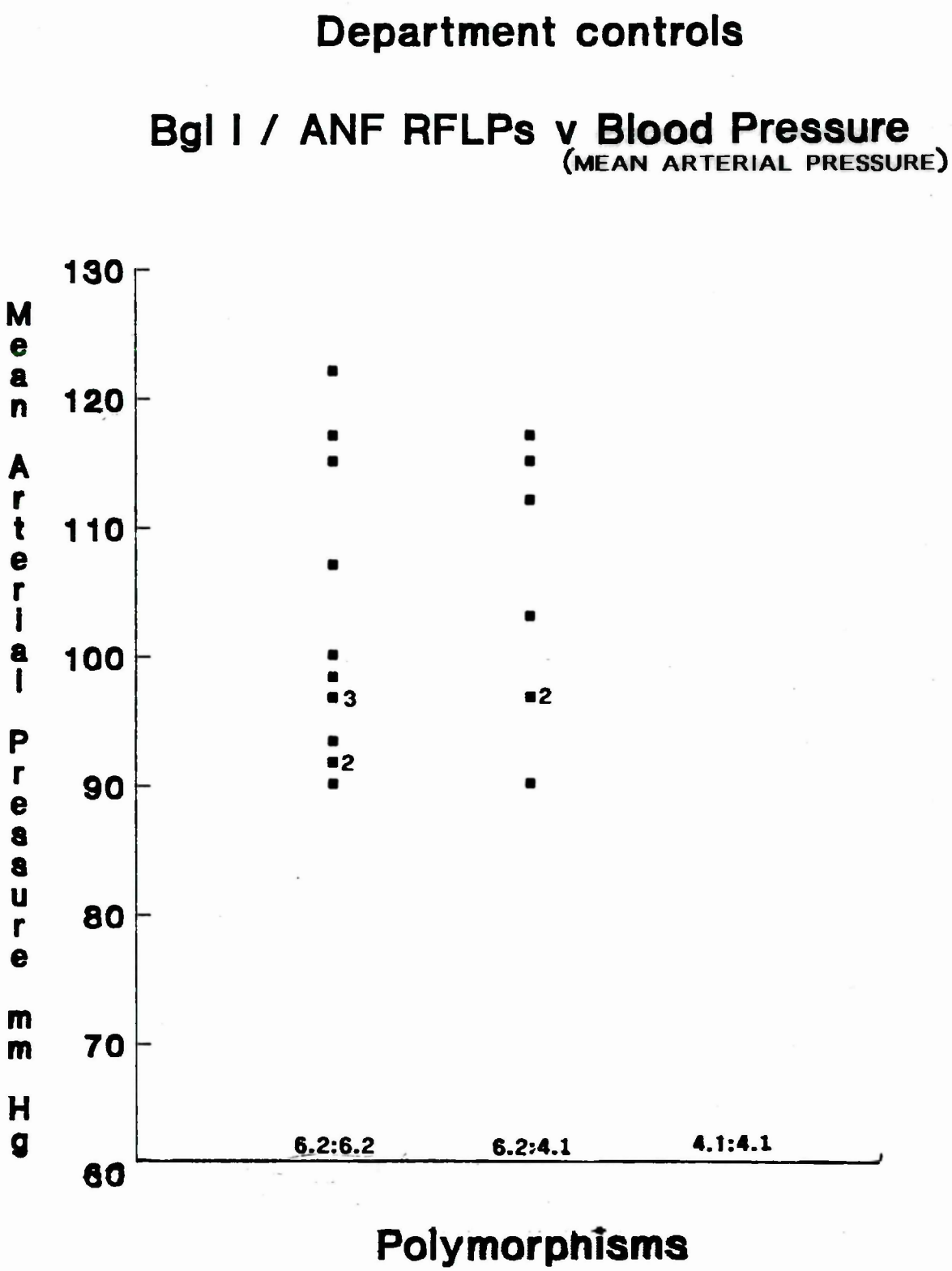

Department controls
Bgl I / ANF RFLPs v Blood Pressure
(MEAN ARTERIAL PRESSURE)
Mean Arterial Presasure mm Hg
130
120
110
100
90
80
70
60
3
2
2
6.2:6.2
6.2:4.1
4.1:4.1
Polymorphisms

FIGURE 20.

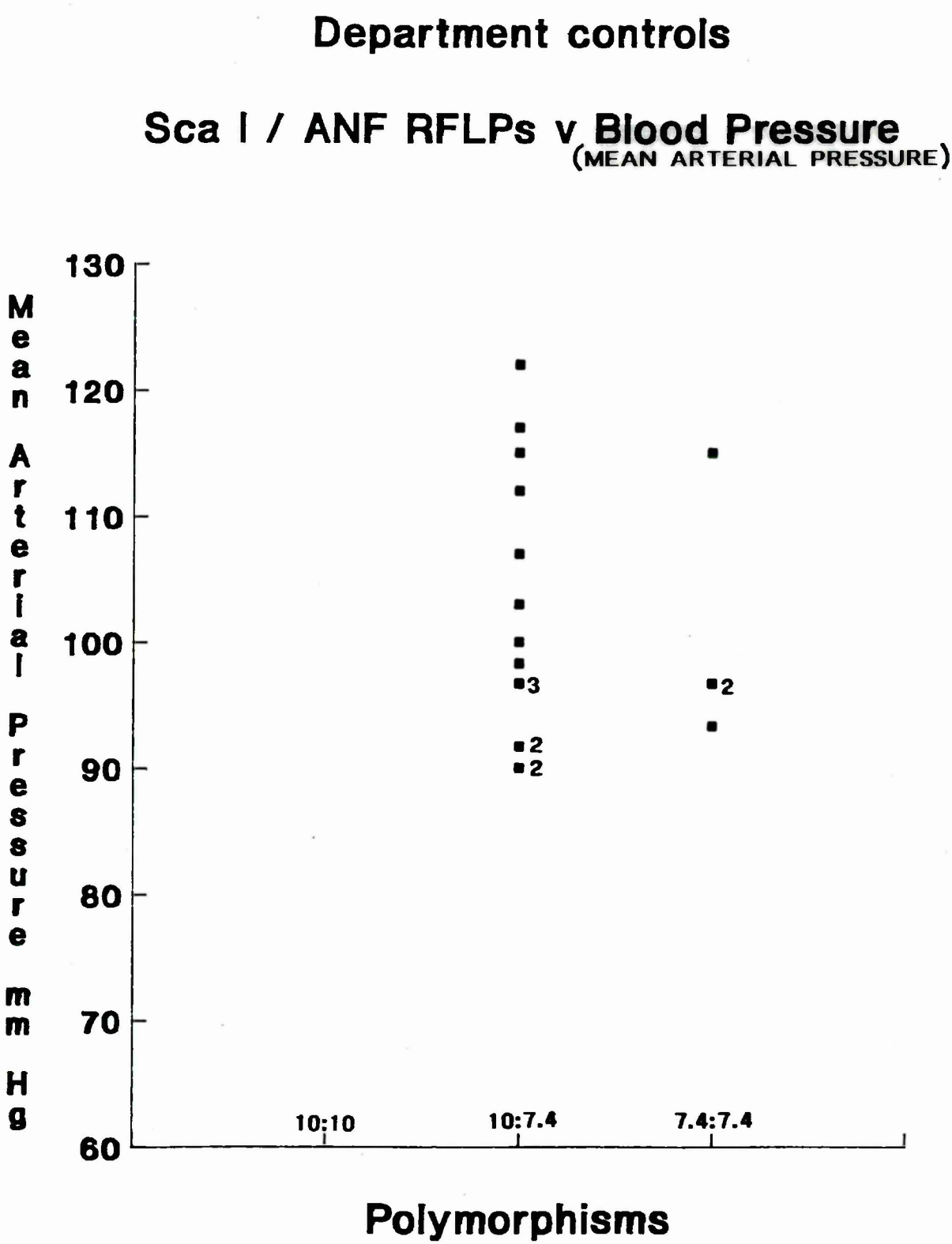

Department controls
Sca I / ANF RFLPs v Blood Pressure
(MEAN ARTERIAL PRESSURE)
Mean Arterial Pressure mm Hg
130
120
110
100
90
80
70
60
10:10
10:7.4
7.4:7.4
Polymorphisms

FIGURE 21.

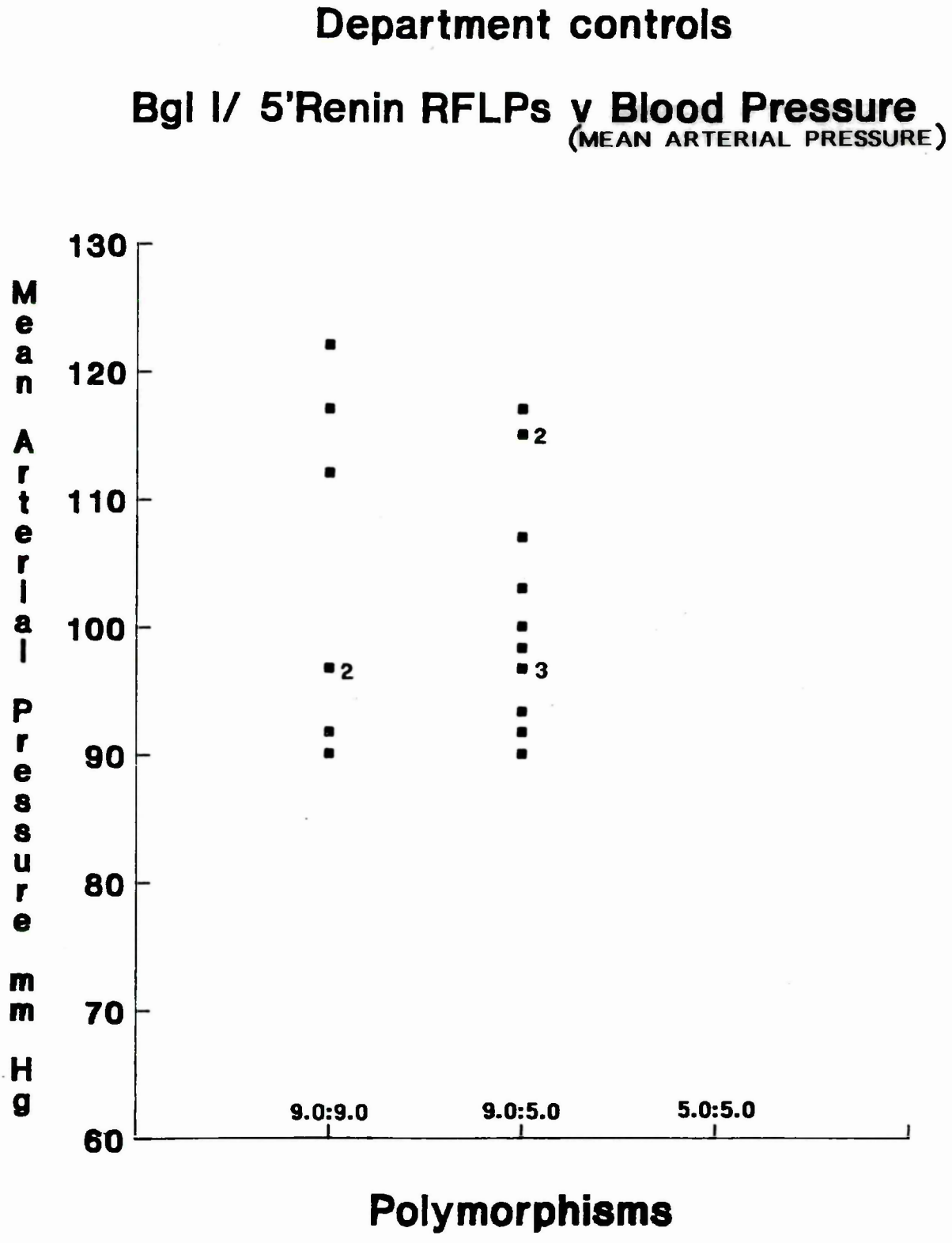

Department controls
Bgl I/ 5'Renin RFLPs v Blood Pressure
(MEAN ARTERIAL PRESSURE)
Mean Arterial Pressure mm Hg
130
120
110
100
90
80
70
60
2
2
3
9.0:9.0
9.0:5.0
5.0:5.0
Polymorphisms

FIGURE 22.

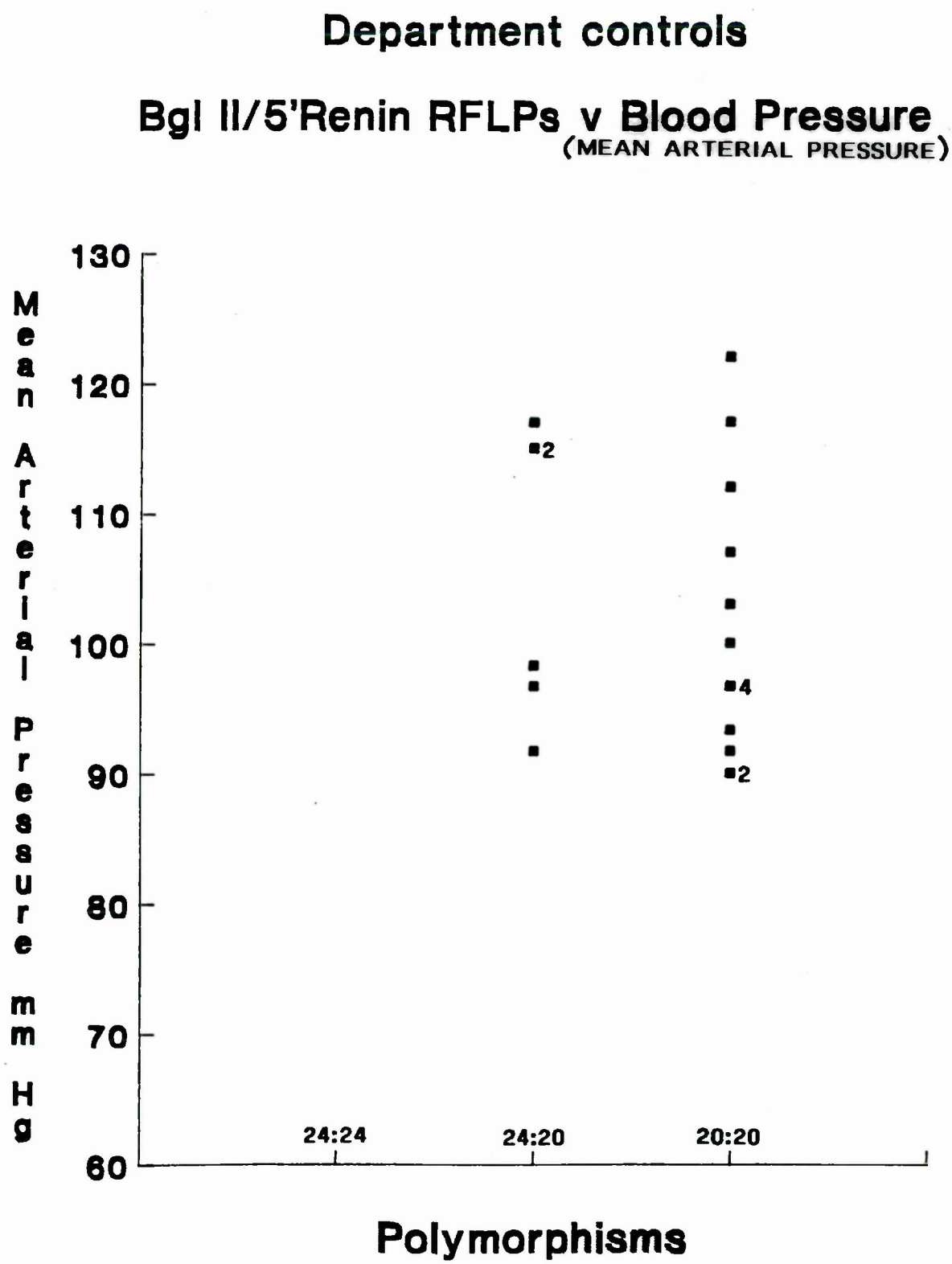

Department controls
Bgl II/5'Renin RFLPs v Blood Pressure
(MEAN ARTERIAL PRESSURE)
Mean Arterial Pressure mm Hg
130
120
110
100
90
80
70
60
2
4
2
24:24
24:20
20:20
Polymorphisms

FIGURE 23.

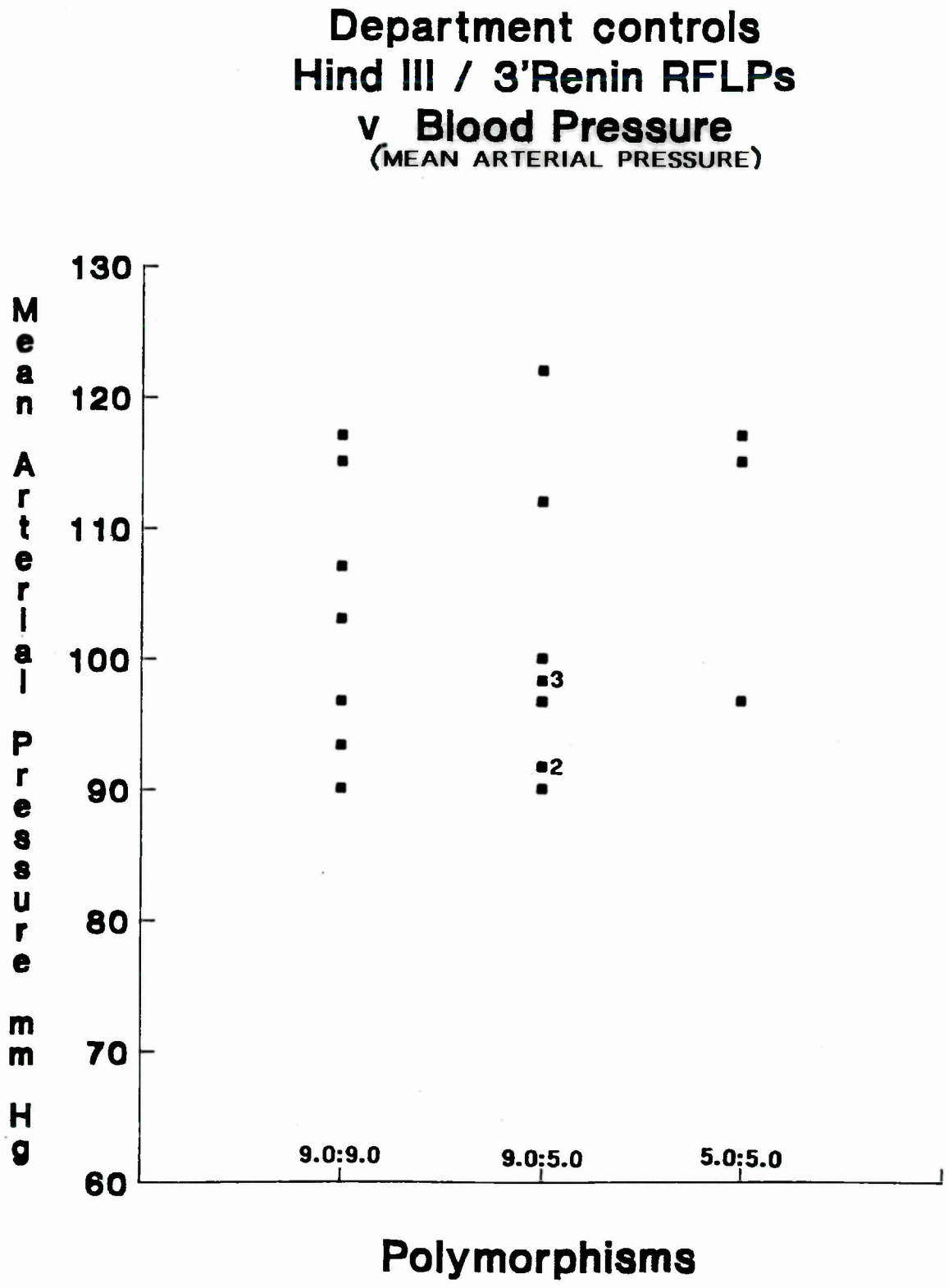
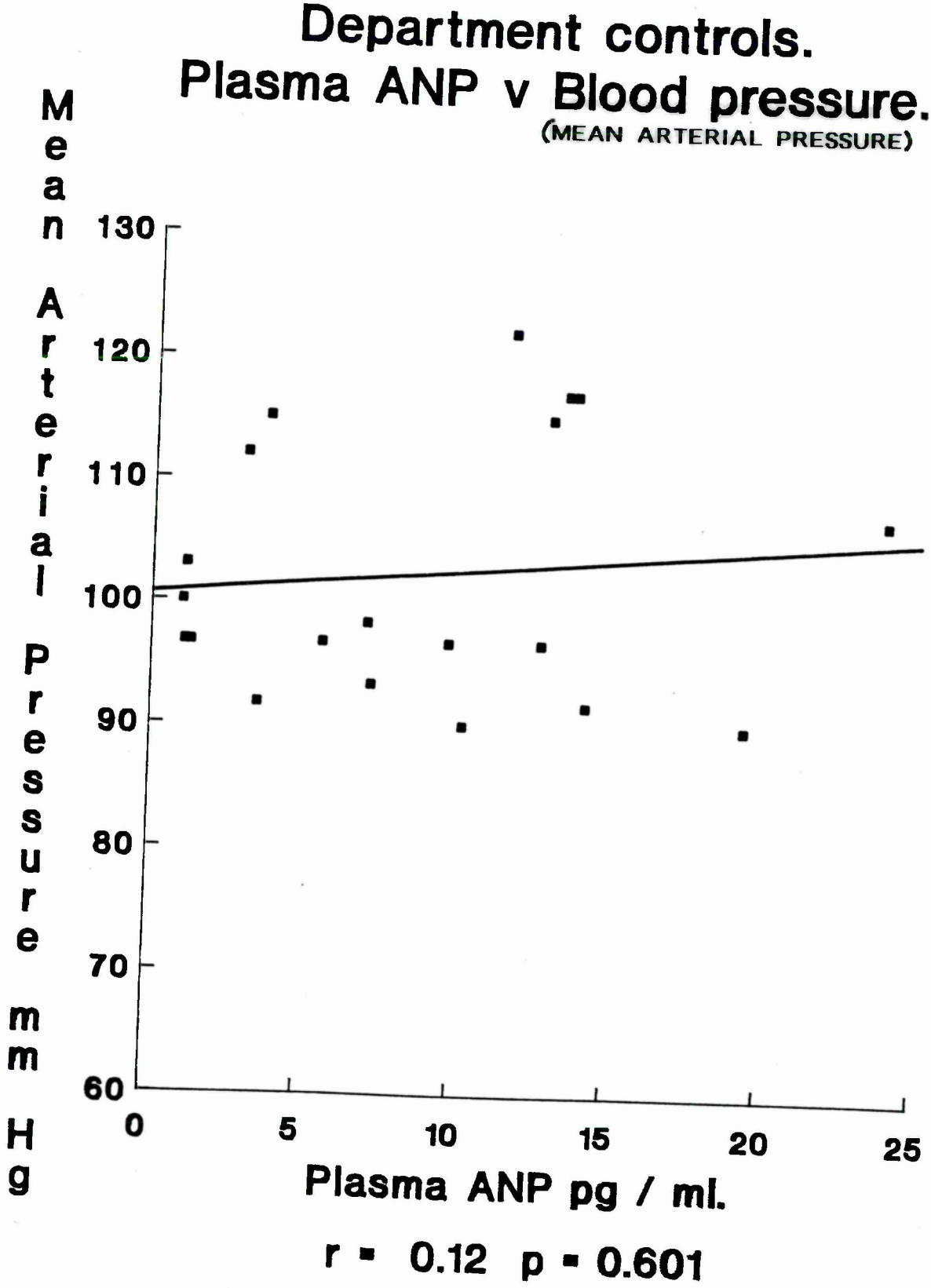
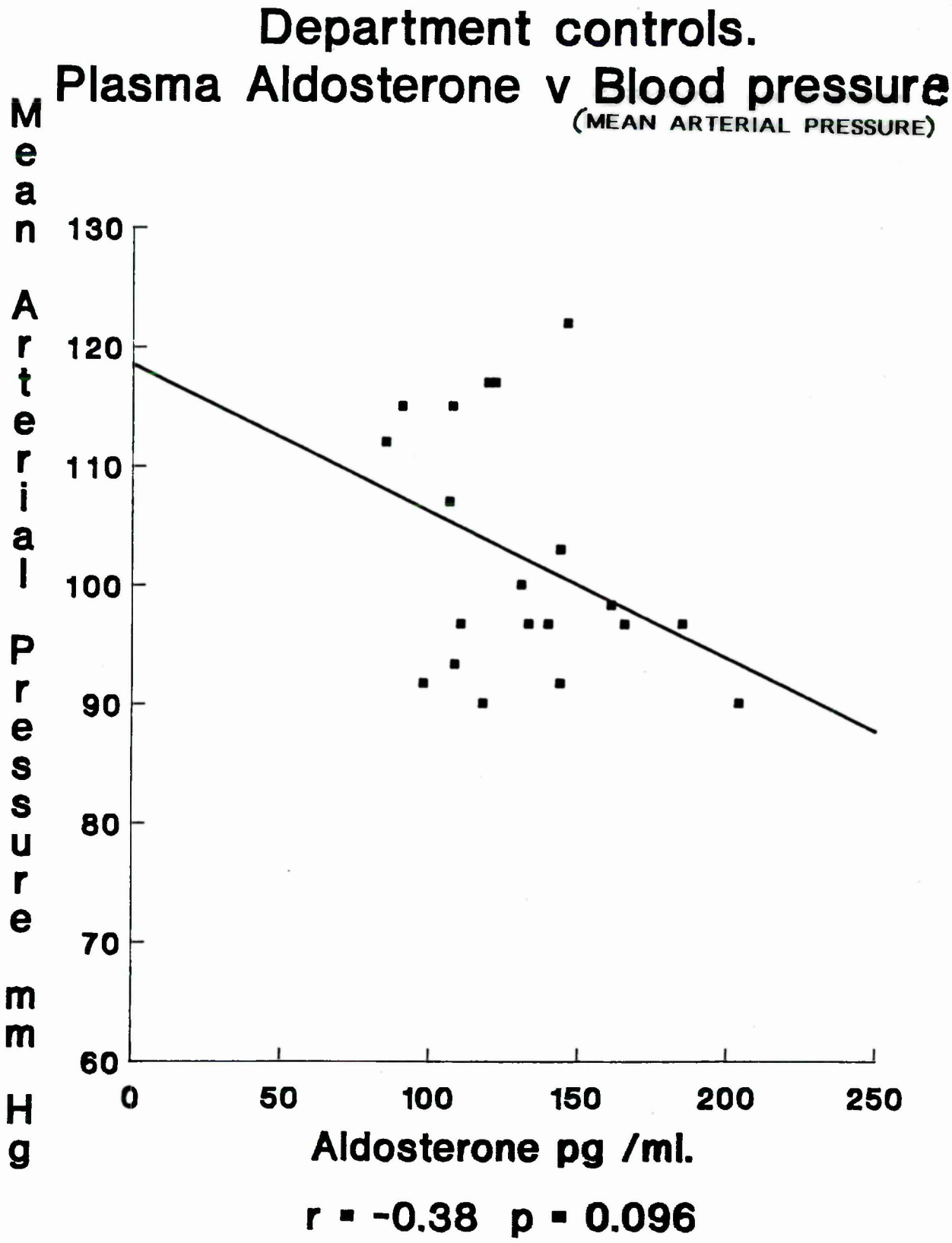

Department controls
Hind III / 3'Renin RFLPs
v Blood Pressure
(MEAN ARTERIAL PRESSURE)
Mean Arterial Pressure mm Hg
130
120
110
100
90
80
70
60
3
2
9.0:9.0
9.0:5.0
5.0:5.0
Polymorphisms

FIGURE 24.



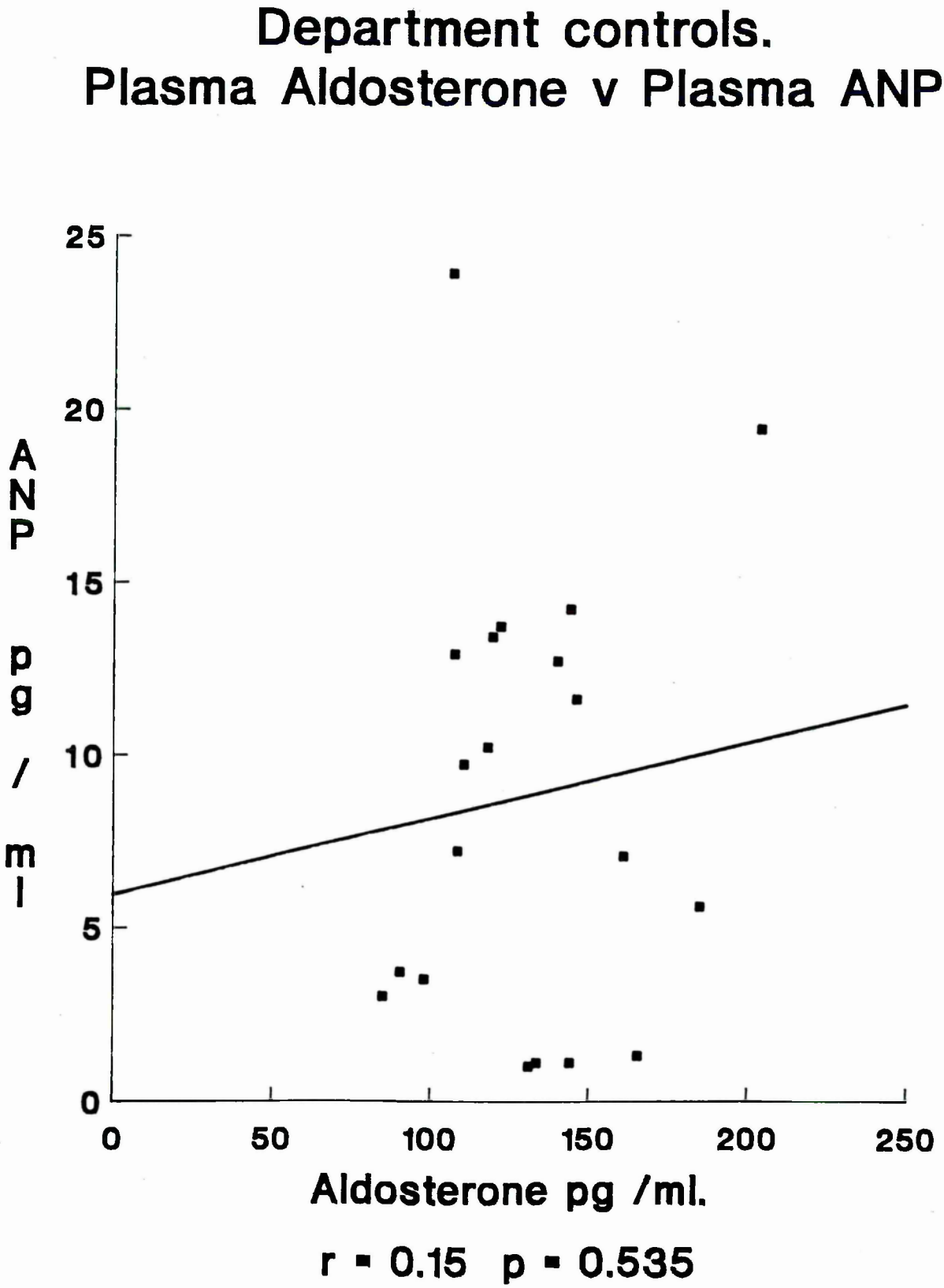
BEST FIT LINE DRAWN BY METHOD OF LEAST SQUARES.

FIGURE 25.



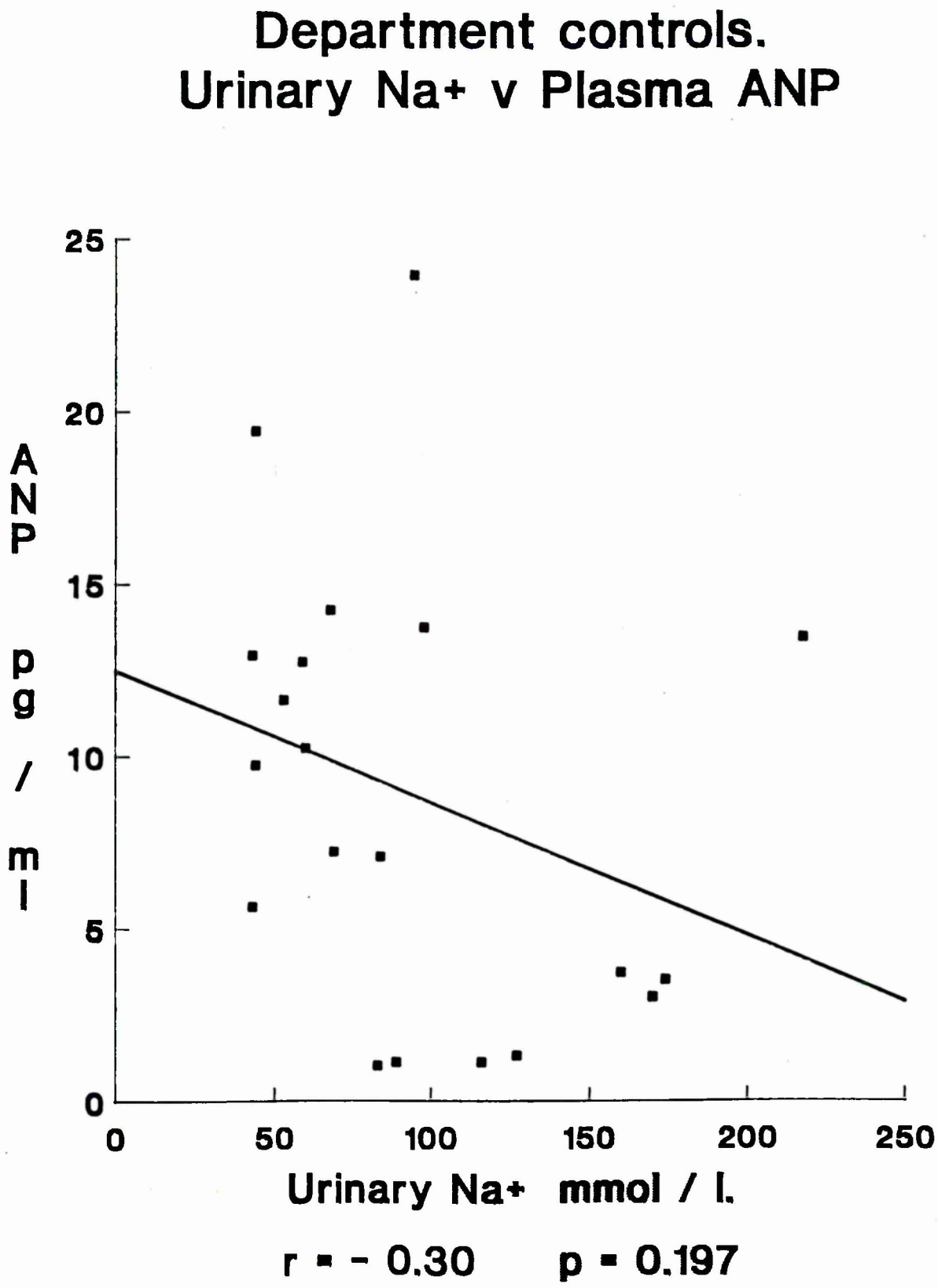
BEST FIT LINE DRAWN BY METHOD OF LEAST SQUARES.

FIGURE 26.



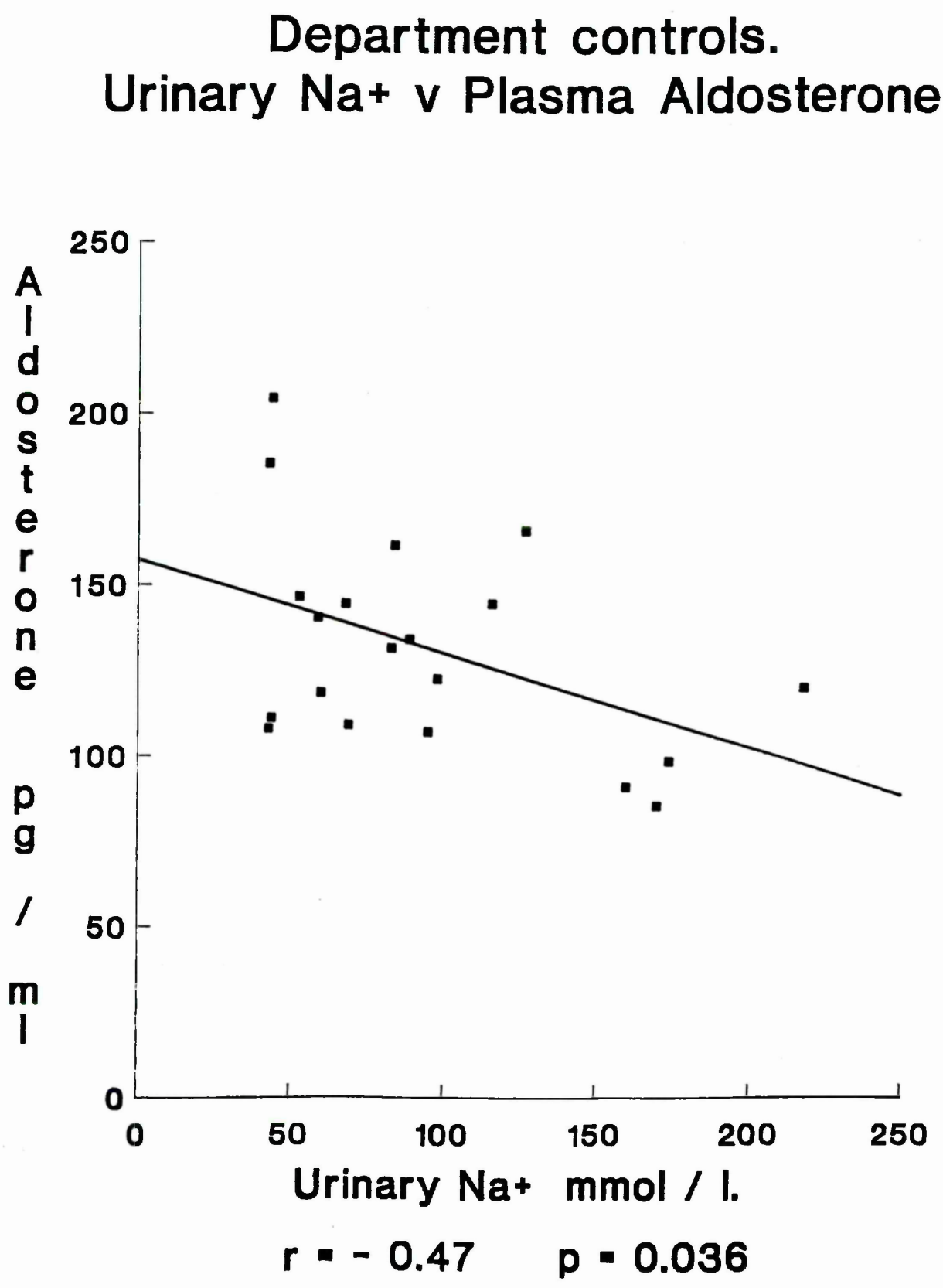
BEST FIT LINE DRAWN BY METHOD OF LEAST SQUARES.

FIGURE 27.



BEST FIT LINE DRAWN BY METHOD OF LEAST SQUARES.

FIGURE 28.



BEST FIT LINE DRAWN BY METHOD OF LEAST SQUARES.

FIGURE 29.

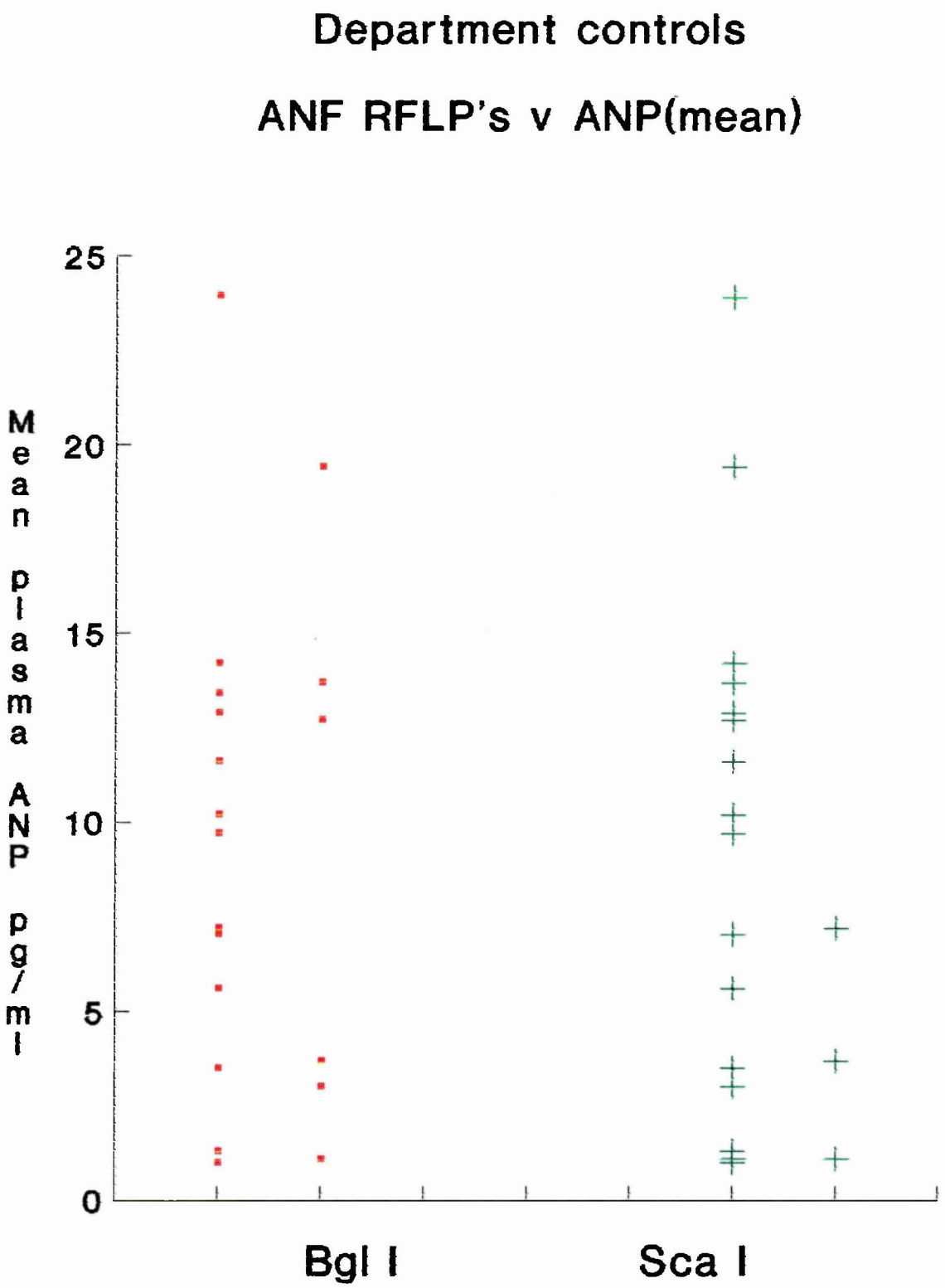

Department controls
ANF RFLP's v ANP(mean)
Mean plasma ANP pg/ml
25
20
15
10
5
0
Bgl I
Sca I

FIGURE 30.

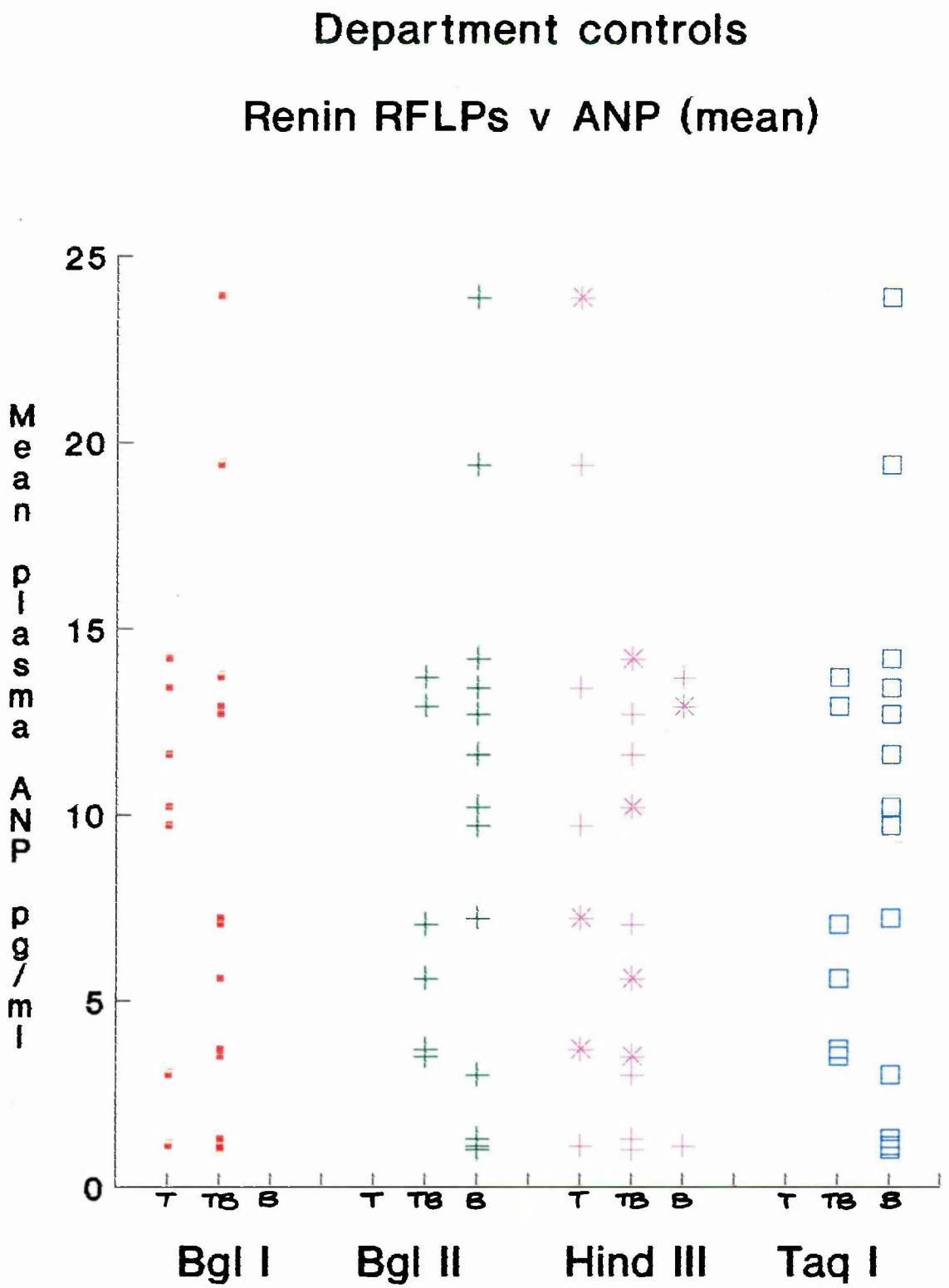

Department controls
Renin RFLPs v ANP (mean)
Mean plasma ANP pg/ml
25
20
15
10
5
0
T
TB
B
Bgl I
Bgl II
Hind III
Taq I

FIGURE 31.

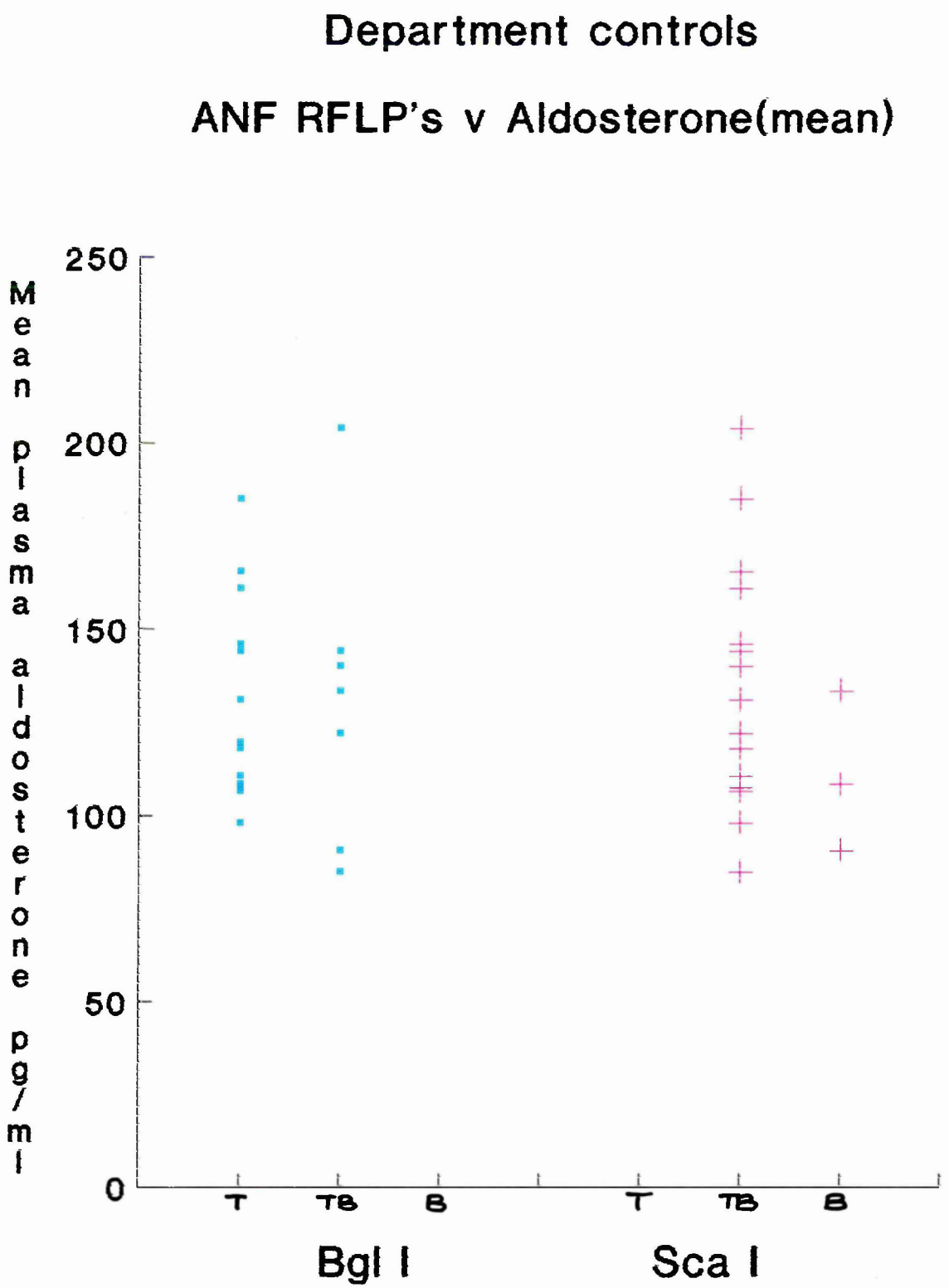
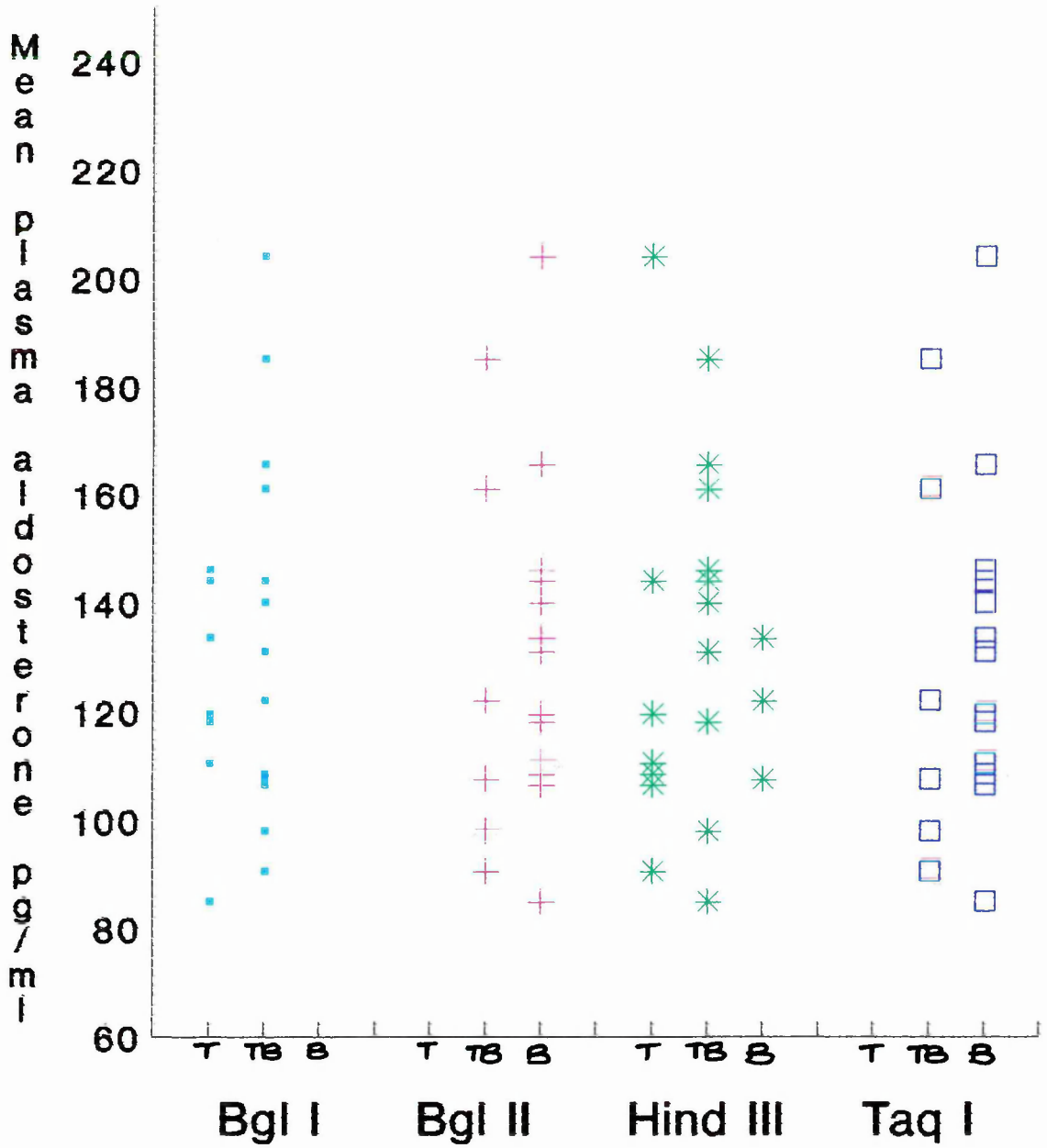

Department controls
ANF RFLP's v Aldosterone(mean)
Mean plasma aldosterone pg/ml
250
200
150
100
50
0
T
TB
B
T
TB
B
Bgl I
Sca I

FIGURE 32.


Department controls
Renin RFLPs v Aldosterone (mean)
Mean plasma aldosterone pg/ml
240
220
200
180
160
140
120
100
80
60
T
TB
B
Bgl I
Bgl II
Hind III
Taq I

Section 4. Study group 3. Caucasian and Afro-caribbean subjects in the upper and lower quintiles of diastolic blood pressure.

TABLE 19.

5' Renin probe on Bgl I digests.

Polymorphism T = 9.0 Kb B = 5.0 Kb

Lower quintile

	9.0:9.0	9.0:5.0	5.0:5.0	Frequency
Afro-Caribbean	8	9	7	0.52/0.48
Caucasian	12	13	2	0.71/0.33

Upper quintile

	9.0:9.0	9.0:5.0	5.0:5.0	Frequency
Afro-Caribbean	4	8	14	0.31/0.69
Caucasian	13	11	1	0.74/0.26

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 18.96$ p = 0.0001

Upper V Lower quintiles $\chi^2 = 1.96$ p = 0.3748

TABLE 20.

5' Renin probe on Taq I digests.

Polymorphism T = 11.0 Kb B = 9.8 Kb

Lower quintile

	11.0:11.0	11.0:9.8	9.8:9.8	Frequency
Afro-Caribbean	3	10	11	0.33/0.67
Caucasian	0	8	19	0.15/0.85

Upper quintile

	11.0:11.0	11.0:9.8	9.8:9.8	Frequency
Afro-Caribbean	1	11	15	0.24/0.76
Caucasian	0	7	18	0.14/0.86

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 6.91$ $p = 0.0316$

Upper V Lower quintiles $\chi^2 = 1.13$ $p = 0.5694$

TABLE 21.

3' Renin probe on Hind III digests.

Polymorphism T = 9.0 Kb B = 6.2 Kb

Lower quintile

	9.0:9.0	9.0:6.2	6.2:6.2	Frequency
Afro-Caribbean	9	14	1	0.67/0.33
Caucasian	15	13	1	0.74/0.26

Upper quintile

	9.0:9.0	9.0:6.2	6.2:6.2	Frequency
Afro-Caribbean	8	16	2	0.62/0.38
Caucasian	12	10	1	0.74/0.26

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 3.36$ $p = 0.1864$

Upper V Lower quintiles $\chi^2 = 0.43$ $p = 0.808$

TABLE 22.

5' Renin probe on Bgl II digests.

Polymorphism T = 24.0 Kb B = 20.0 Kb

Lower quintile

	24.0:24.0	24.0:20.0	20.0:20.0	Frequency
Afro-Caribbean	3	12	9	0.37/0.63
Caucasian	0	7	20	0.13/0.87

Upper quintile

	24.0:24.0	24.0:20.0	20.0:20.0	Frequency
Afro-Caribbean	4	10	13	0.33/0.67
Caucasian	0	6	19	0.12/0.88

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 14.04$ $p = 0.0009$

Upper V Lower quintiles $\chi^2 = 0.54$ $p = 0.7642$

TABLE 23.

ANF probe on Bgl I digests.

Polymorphism T = 6.2 Kb B = 4.1 Kb

Lower quintile

	6.2:6.2	6.2:4.1	4.1:4.1	Frequency
Afro-Caribbean	6	12	6	0.50/0.50
Caucasian	20	7	0	0.87/0.13

Upper quintile

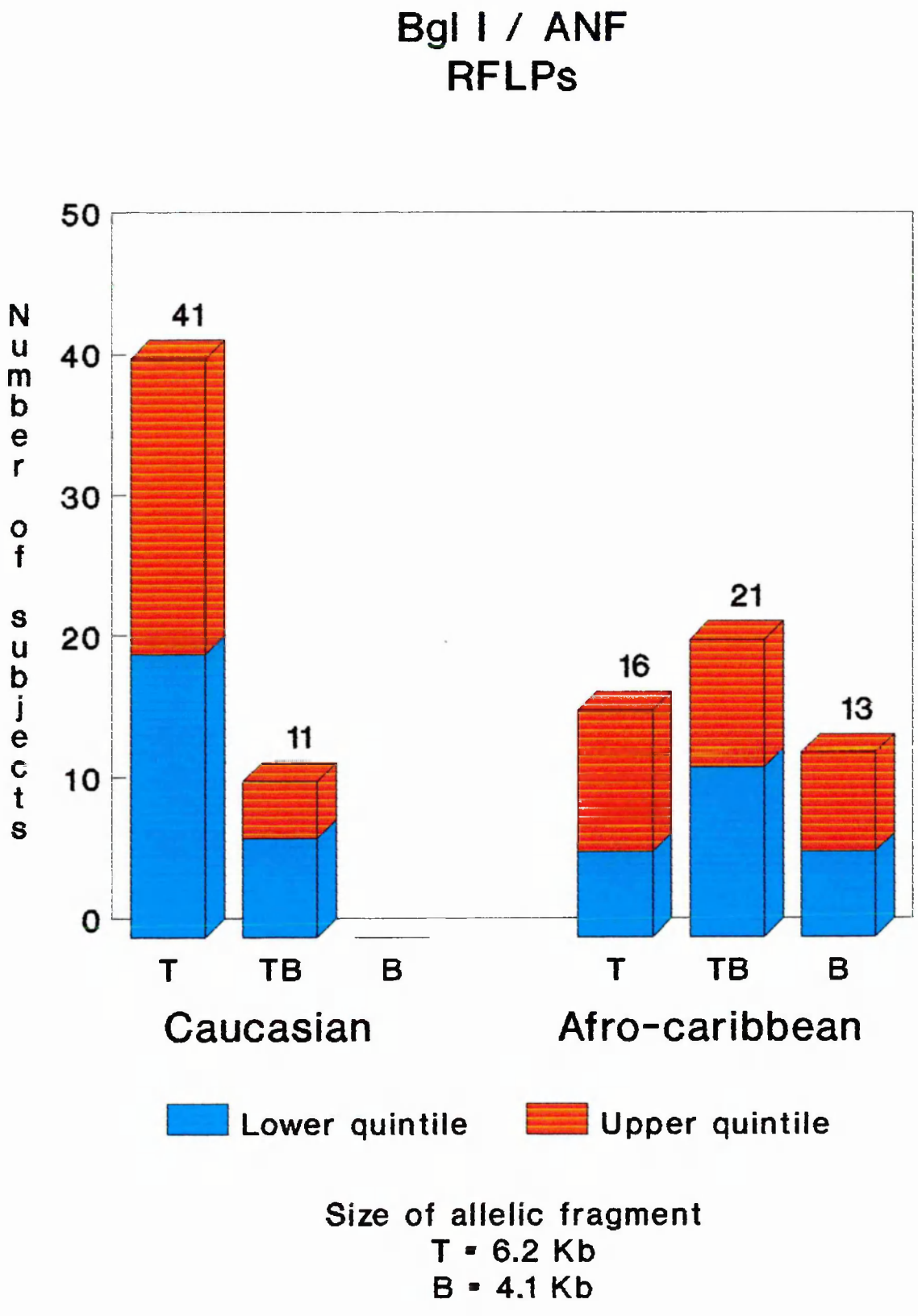
	6.2:6.2	6.2:4.1	4.1:4.1	Frequency
Afro-Caribbean	10	9	7	0.56/0.44
Caucasian	21	4	0	0.92/0.08

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 27.06$ $p = <0.0001$

Upper V Lower quintiles $\chi^2 = 1.64$ $p = 0.4403$

FIGURE 33. BLOOD PRESSURE STUDY GROUP. BGL I/ANF



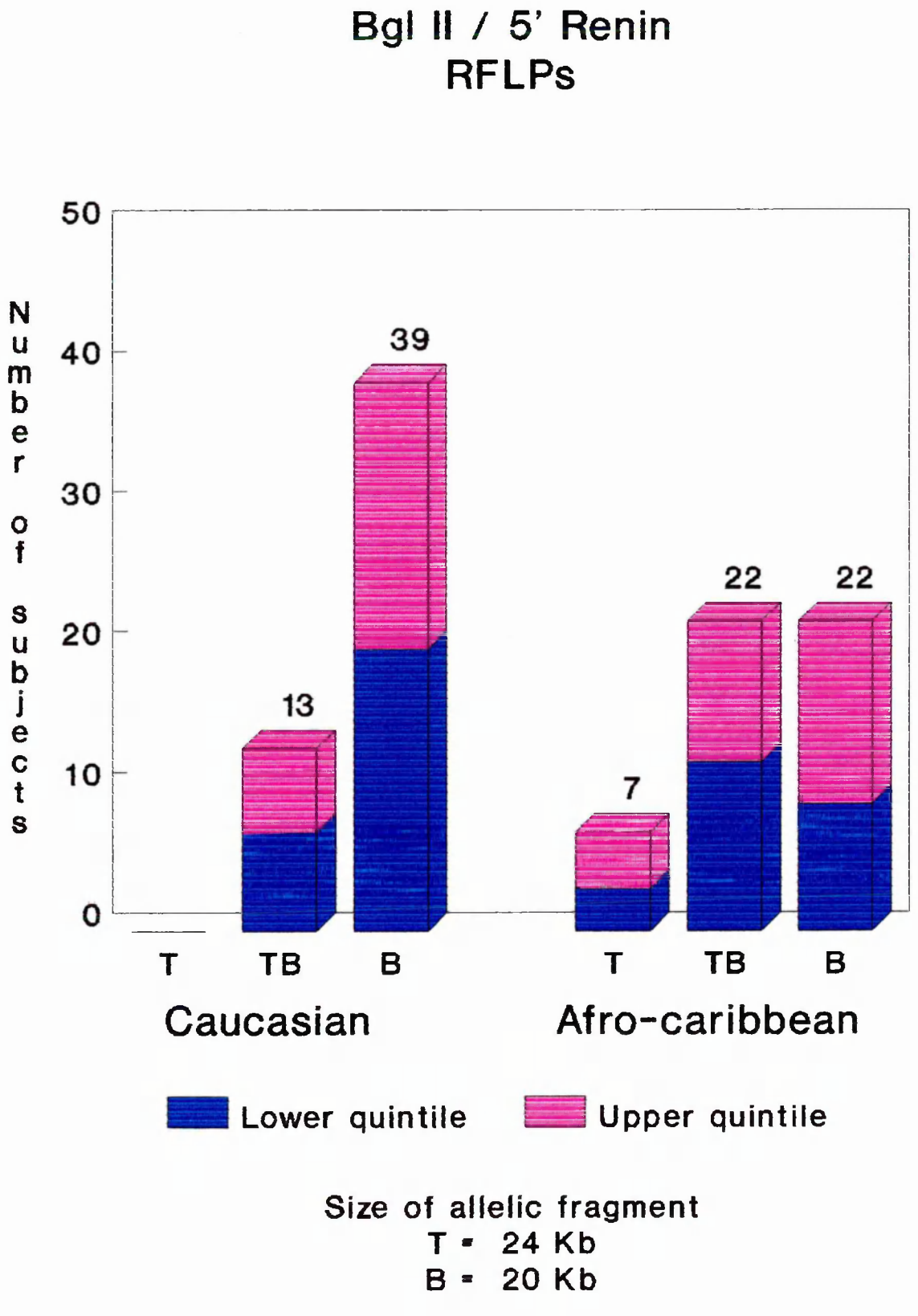

Bgl II / 5' Renin
RFLPs
Number of subjects
50
40
30
20
10
0
39
13
22
22
7
T
TB
B
T
TB
B
Caucasian
Afro-caribbean
Lower quintile
Upper quintile
Size of allelic fragment
T = 24 Kb
B = 20 Kb

FIGURE 35. BLOOD PRESSURE STUDY GROUP. TAQ I/5'RENIN.

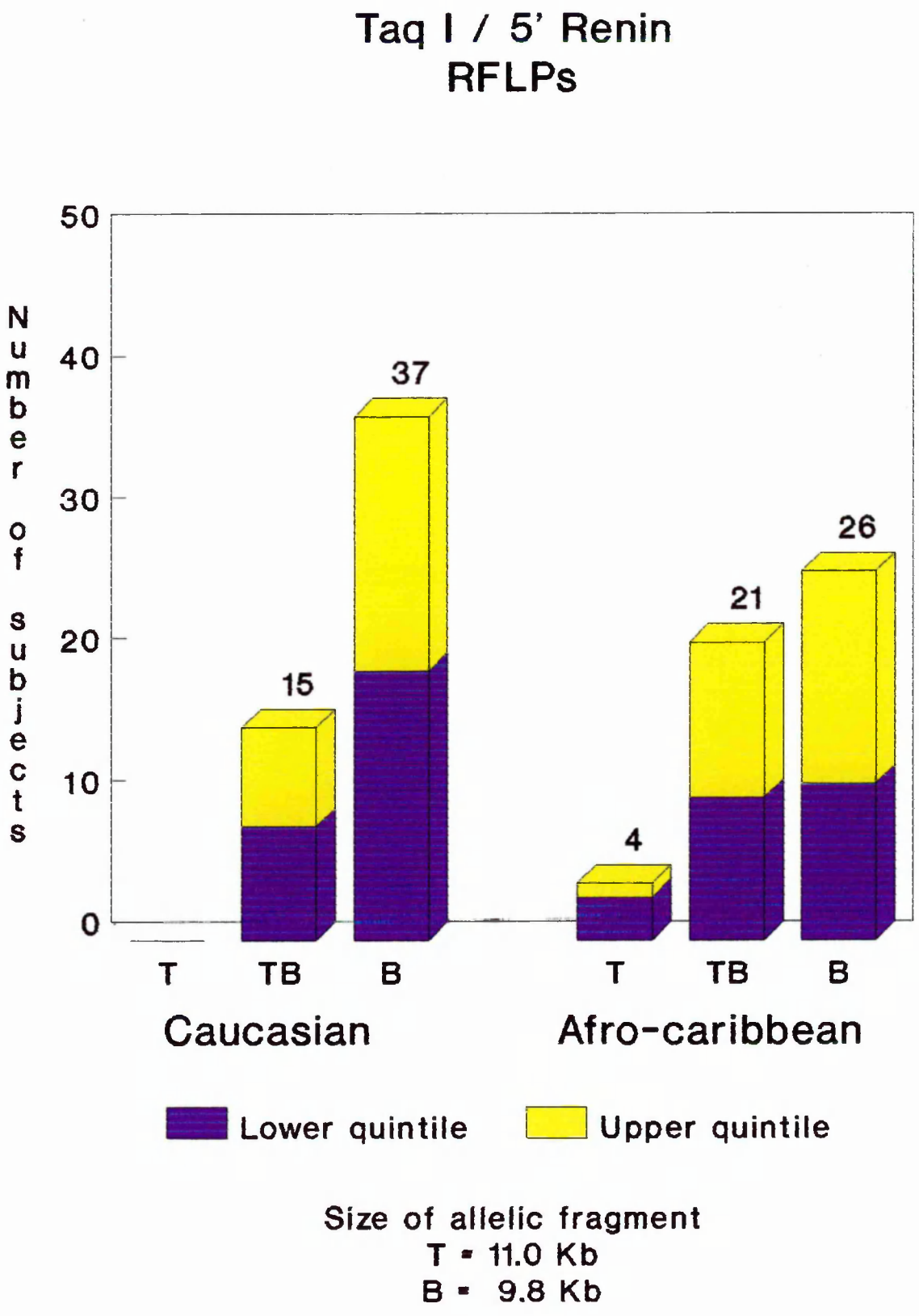


FIGURE 36. BLOOD PRESSURE STUDY GROUP. HIND III/3'RENIN.

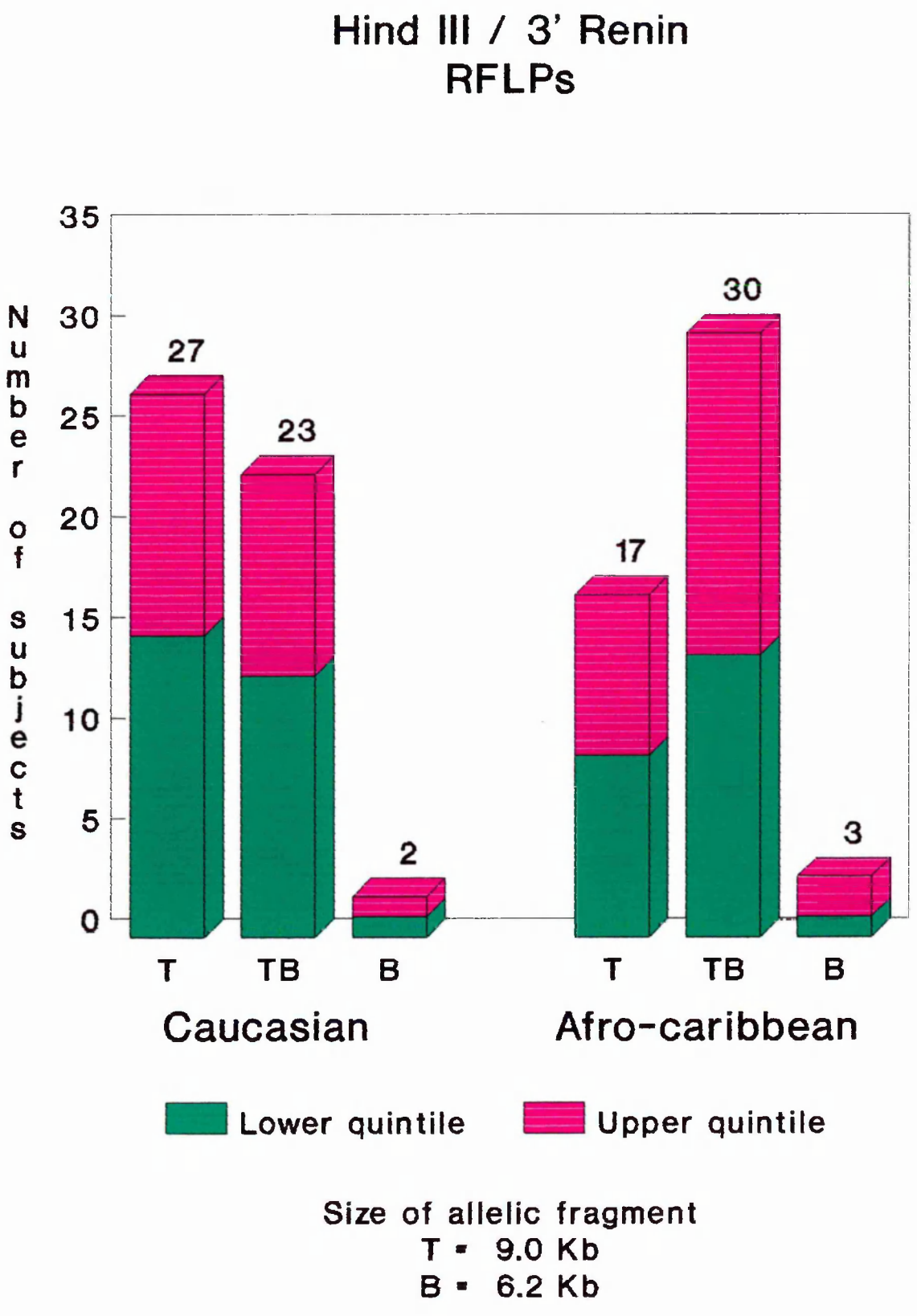


FIGURE 37. BLOOD PRESSURE STUDY GROUP. BGL I/5'RENIN

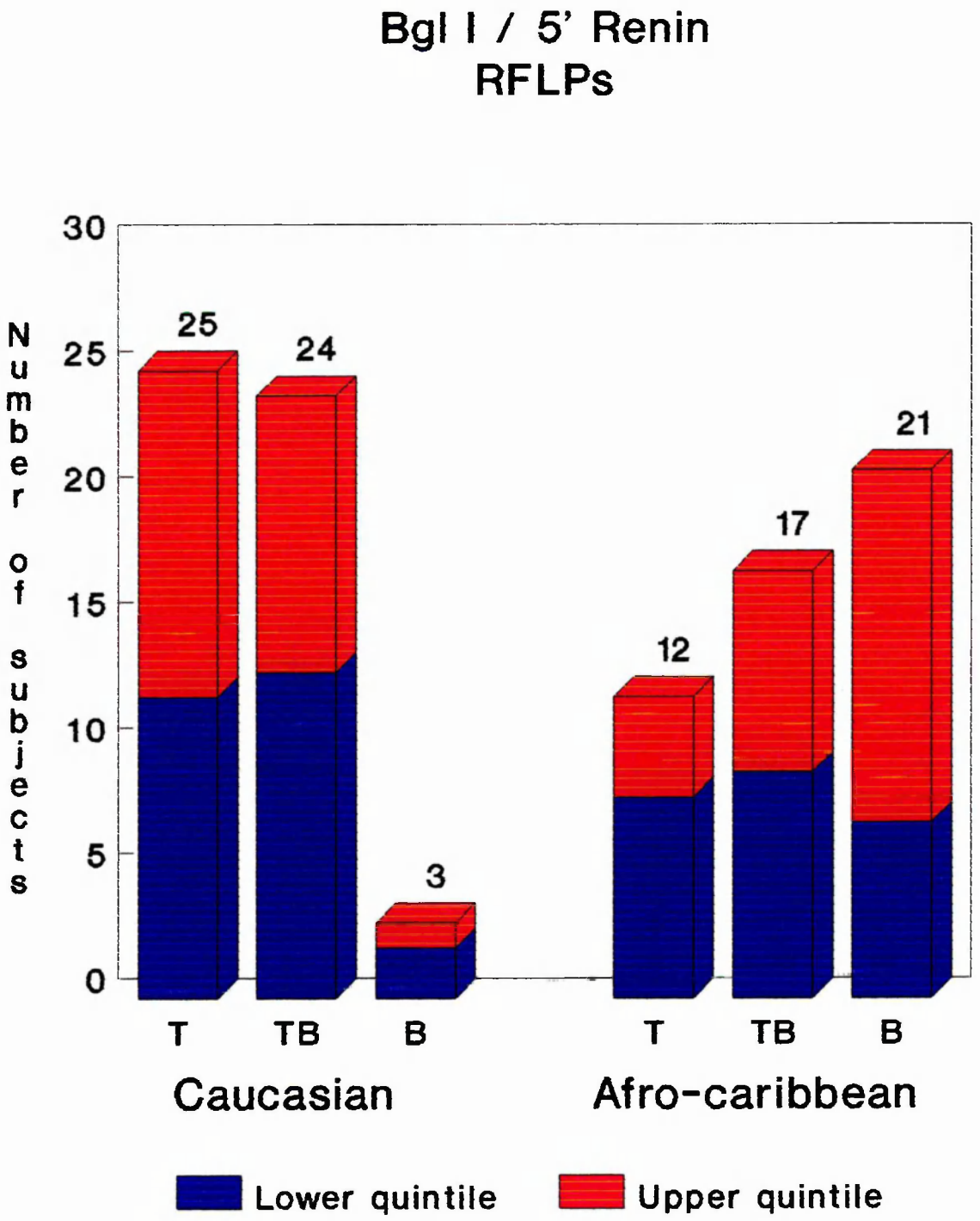


FIGURE 38. PHOTOGRAPH OF AUTORADIOGRAPH OF HIND III GENOMIC DIGEST PROBED WITH 3'RENIN.

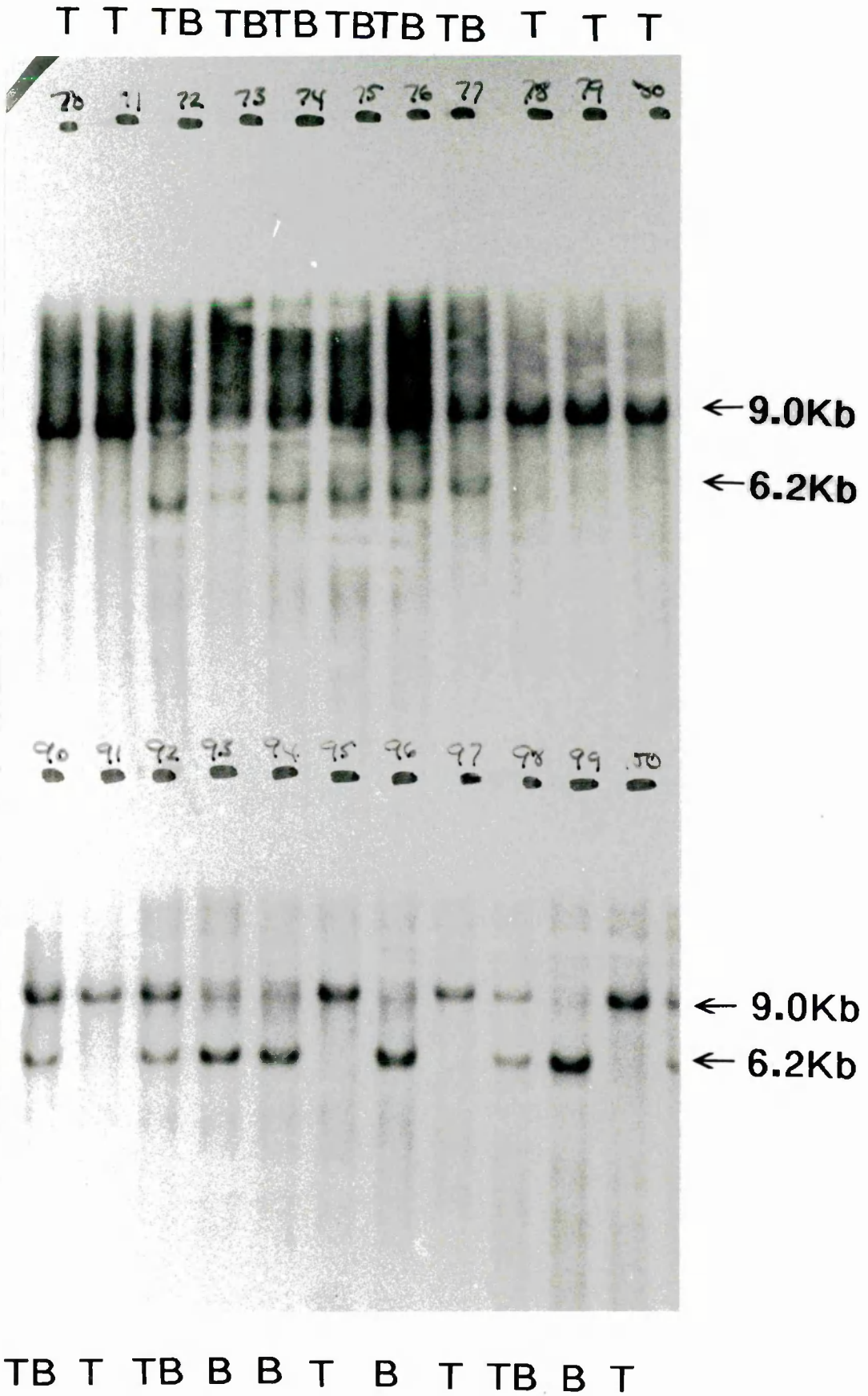


FIGURE 39. PHOTOGRAPH OF AUTORADIOGRAPH OF BGL II GENOMIC DIGESTS PROBED WITH 5'RENIN.

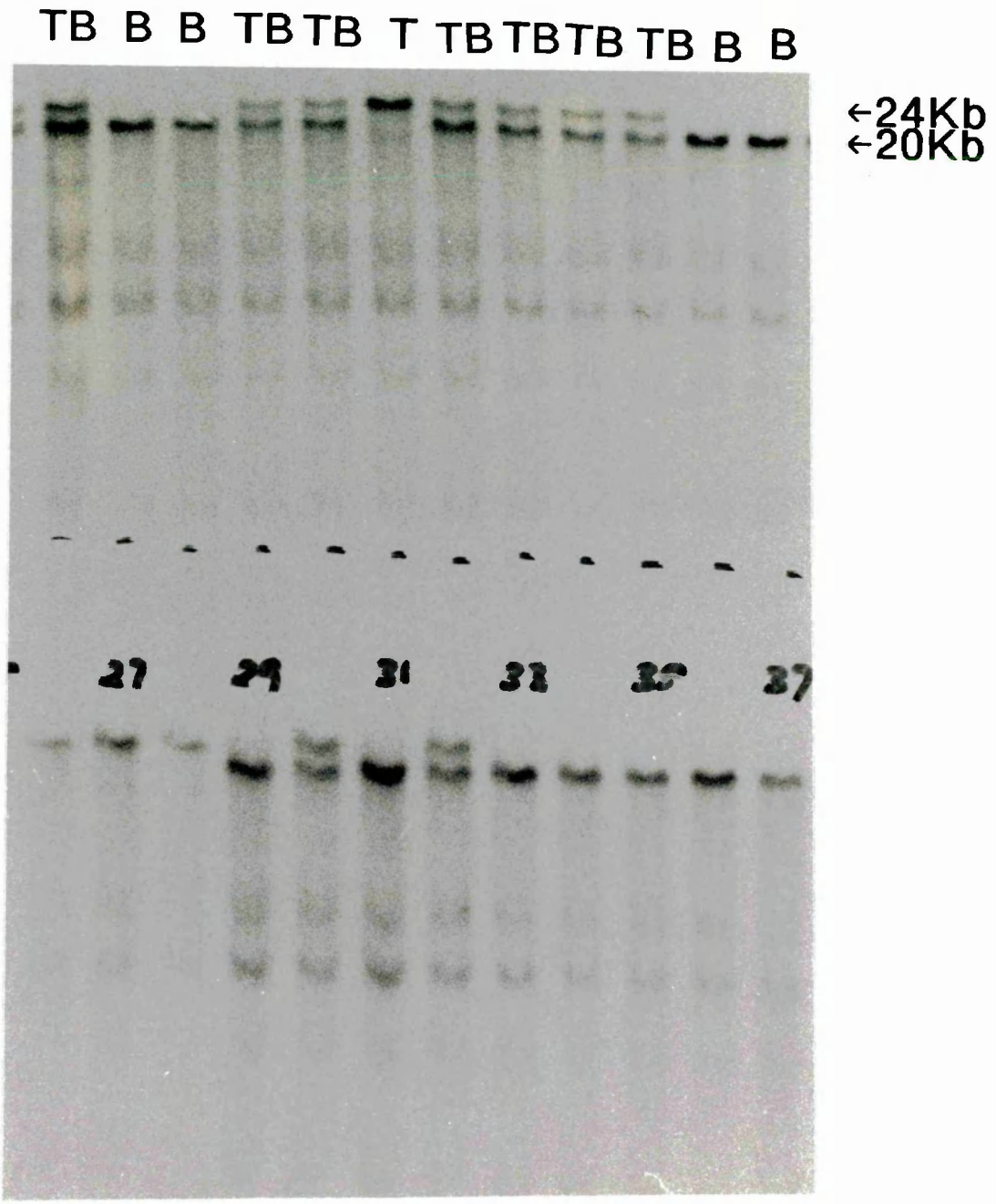


TABLE 24. PLASMA ACTIVE RENIN CONCENTRATIONS.

RFLPs and Renin concentration

Allelic polymorphism	PARC uU/ml.	95% confidence limits.
BGL I / 5'RENIN		
9.0:9.0	25.5	(17.7 - 36.8)
9.0:5.0	14.9	(10.2 - 21.7)
5.0:5.0	10.1	(5.0 - 20.3)
TAQ I / 5'RENIN		
11.0:11.0	21.5	(10.0 - 46.5)
11.0: 9.8	12.6	(8.3 - 19.1)
9.8: 9.8	22.4	(16.2 - 31.0)
HIND III/3'RENIN		
9.0:9.0	23.8	(14.6 - 38.9)
9.0:6.2	17.0	(12.2 - 23.6)
6.2:6.2	17.0	(6.8 - 42.4)
BGL II/ 5'RENIN		
24.0:24.0	21.5	(10.0 - 46.5)
24.0:20.0	12.6	(8.3 - 19.1)
20.0:20.0	22.4	(16.2 - 31.0)
BGL I / ANF		
6.2:6.2	20.5	(14.8 - 28.4)
6.2:4.1	20.7	(13.3 - 32.2)
4.1:4.1	6.9	(3.1 - 15.2)

Plasma active Renin concentration (PARC)

FIGURE 40. BLOOD PRESSURE STUDY. RFLPS V RENIN.

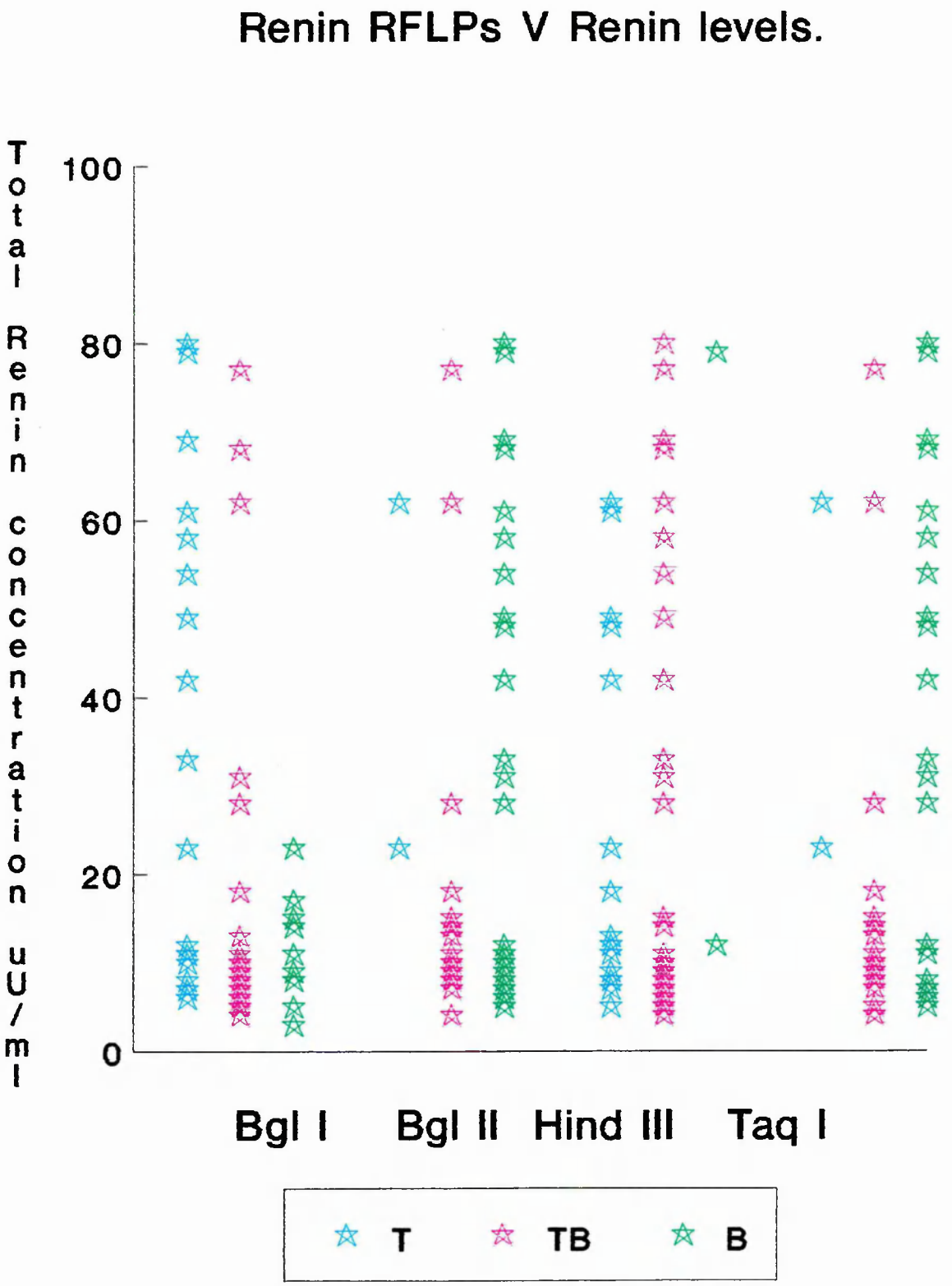


Figure 41. BLOOD PRESSURE STUDY GROUP.

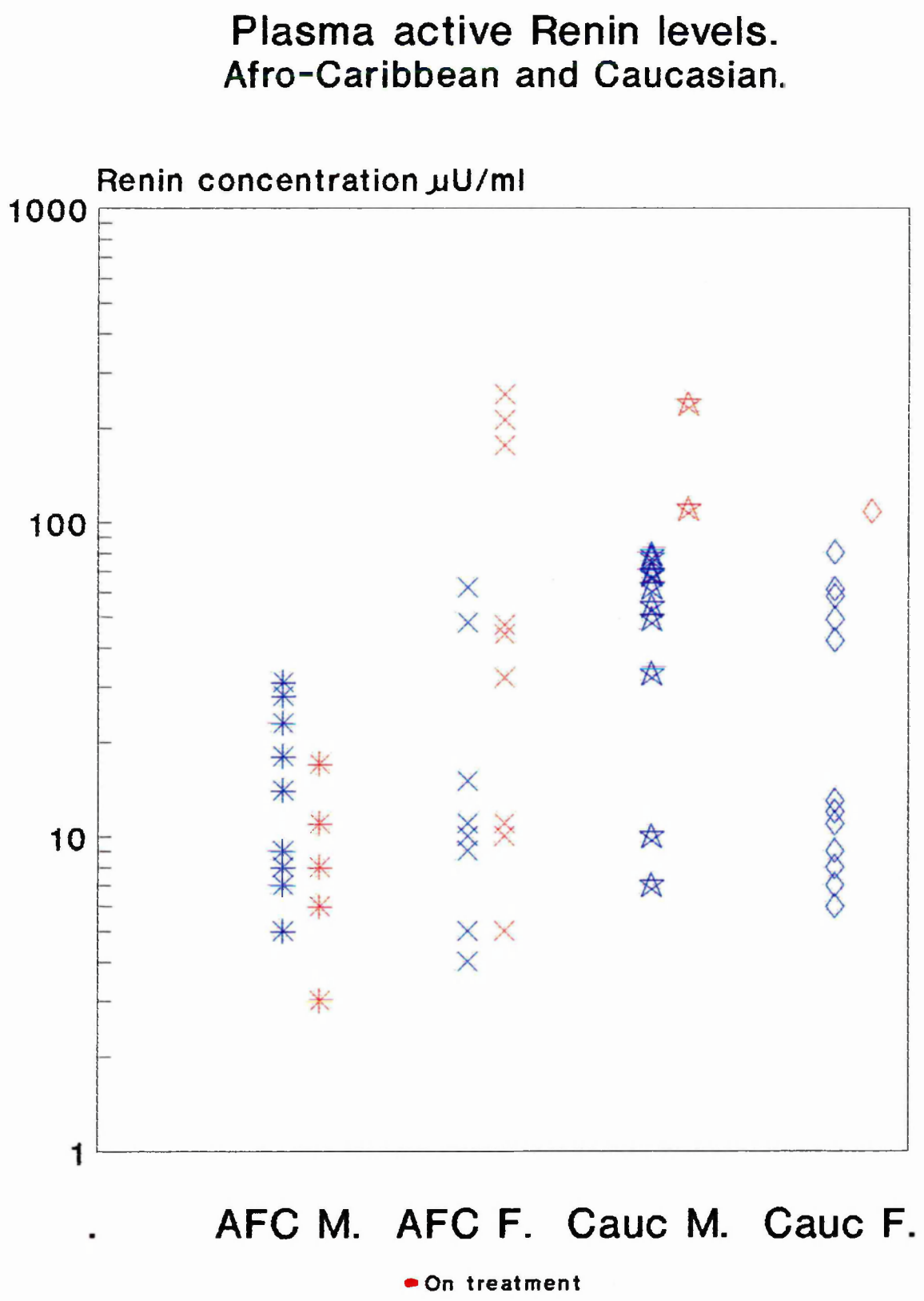


TABLE 25. BLOOD PRESSURE IN BPS.

Blood Pressure Study group.

AFRO-CARIBBEAN

Sex	Quintile	No.	Age	SBP	DBP	MAP	On Treatment
M	U	13	58±1	160±4	98±2	119±2	8
	L	13	59±2	123±4	73±1	90±2	0
F	U	14	60±2	154±6	92±3	112±3	10
	L	11	54±1	110±3	68±2	82±2	0
	All	51	58±1	139±4	84±2	102±2	18

CAUCASIAN

M	U	11	65±2	152±7	89±3	110±4	2
	L	13	61±2	108±2	62±2	77±1	0
F	U	14	62±2	143±5	85±3	105±3	4
	L	14	57±2	104±2	61±2	76±2	0
	All	52	61±1	126±4	74±2	92±3	6

Glucocorticoid receptor (GCR) RFLPs.

The glucocorticoid receptor gene was cloned and made available for research study during the latter part of the project and was studied using the blood pressure study group.

Table 26.

Blood pressure study group.

GCR probe on BCl I digests.

Polymorphism T = 3.7 Kb B = 2.1 Kb

Lower quintile

	3.7:3.7	3.7:2.1	2.1:2.1	Frequency
Afro-Caribbean	5	5	14	0.31/0.69
Caucasian	6	11	7	0.48/0.52

Upper quintile

	3.7:3.7	3.7:2.1	2.1:2.1	Frequency
Afro-Caribbean	2	4	16	0.17/0.83
Caucasian	5	10	7	0.44/0.56

Chi-square test for difference.

Afro-Caribbean V Caucasian $\chi^2 = 11.51$ $p = 0.0032$

Upper V Lower quintiles $\chi^2 = 0.94$ $p = 0.6247$

Log-linear models.

A series of log-linear models were fitted to the GCR / BCl I RFLP data and in order to evaluate which of these models fit the data the G^2 statistic was calculated. The smaller the G^2 statistic the better the model fits the data. The [B] [E] [R] model was the simplest model that was fitted where [B] was blood pressure, [E] was ethnic group and [R] was RFLP profile. Other terms added to this model were associations between two of the variables, i.e. [RE] was the model term for an association between ethnic group and RFLP profile.

Model	G^2	d.f.	P value.	
[B] [E] [R]	13.32	7	0.0646	
[R] [BE]	13.32	6	0.0382	*
[B] [RE]	1.54	5	0.9087	
[E] [RB]	12.38	5	0.0300	*
[BE] [RE] [RB]	0.52	2	0.7720	

Both models denoted by * have a significant lack of fit.

The differences in G^2 between the models with a single association i.e. [B] [RE] and the null model [B] [E] [R] are shown below and these were used to assess whether there were significant associations between the variables.

Model term	G^2	d.f.	P value
[GE]	11.78	2	**0.0028**
[GB]	0.94	2	0.6250
[BE]	0.00	1	1.0000

There was a significant association between RFLP profile and ethnic group [RE] (P= 0.0028) and this was shown in the table of observed frequencies where the Caucasians have a higher proportion of TB while the Afro-Caribbeans have a higher proportion of B. The RFLP profile and blood pressure quintile were independent.

FIGURE 42. BLOOD PRESSURE STUDY GROUP. GCR / BCL I.

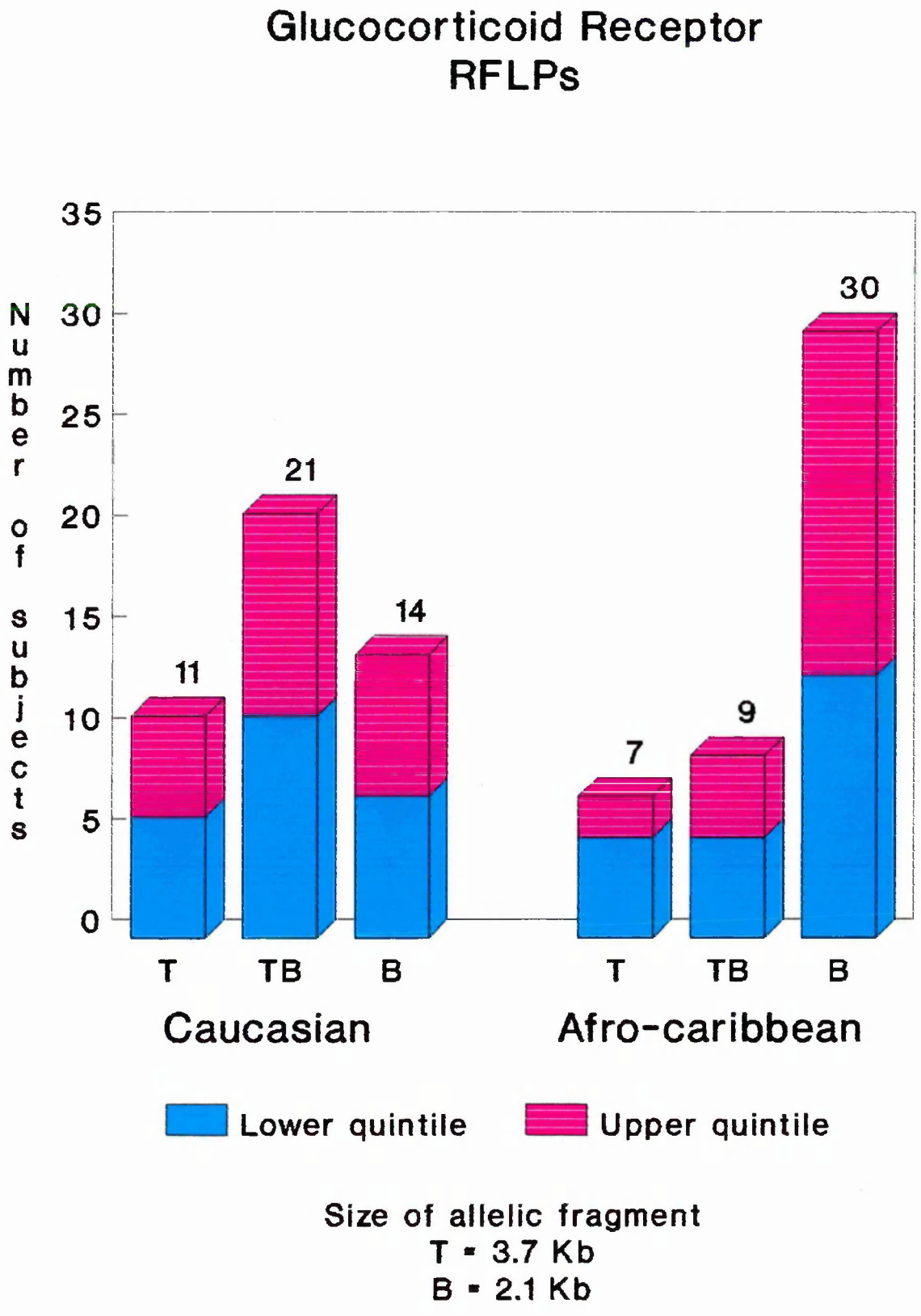
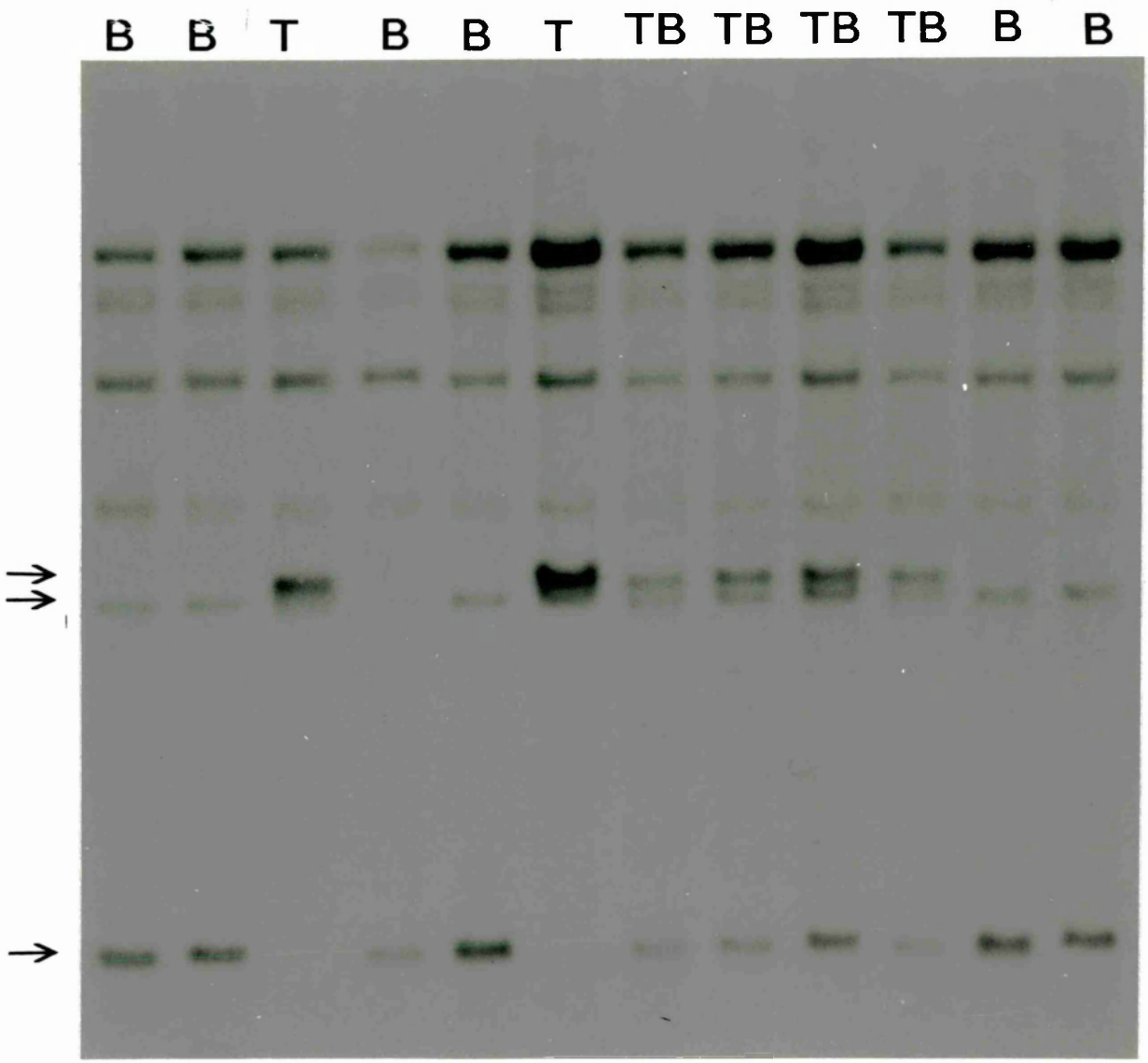


FIGURE 43. PHOTOGRAPH OF AUTORADIOGRAPH OF BCL I GENOMIC DIGESTS PROBED WITH GCR.



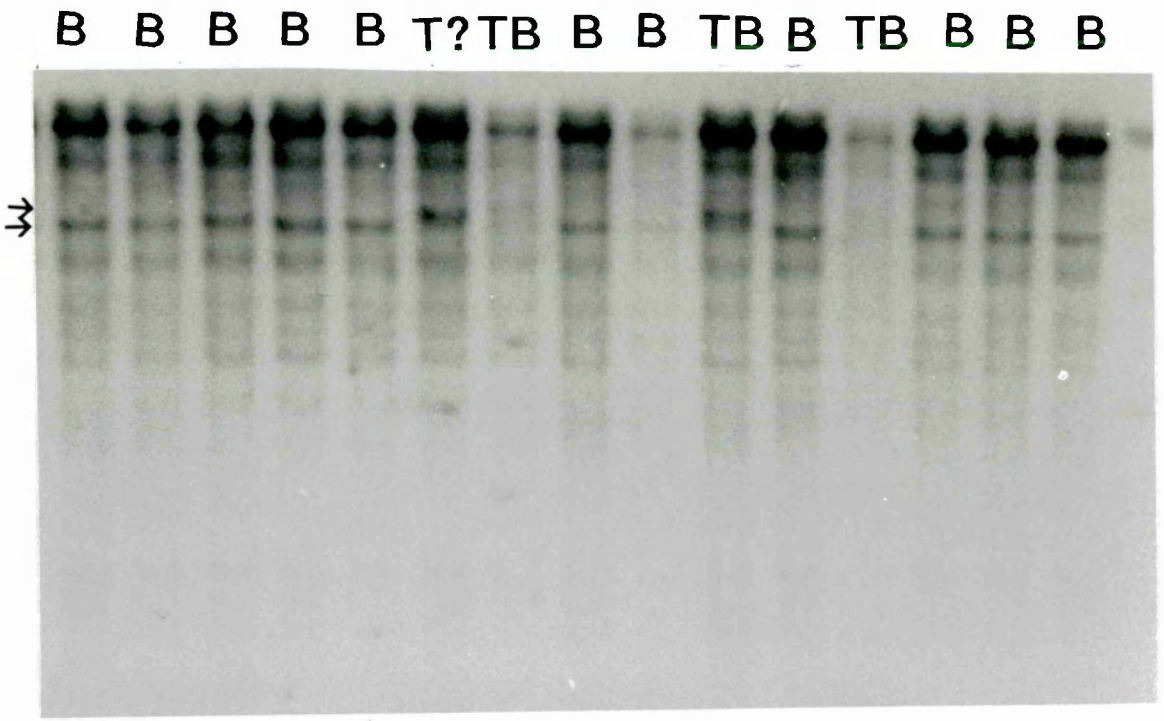
T= 3.7Kb B= 2.1Kb

Kallikrein gene probe.

The kallikrein gene probe was received at the end of the research study and the polymorphism was determined only on a few subjects .

Polymorphism T = > 12.0 Kb B = 9.5 Kb

FIGURE 44. PHOTOGRAPH OF AN AUTORADIOGRAPH OF ECO R I GENOMIC DIGESTS PROBED WITH KALLIKREIN.



T= >12Kb B= 9.5Kb

CHAPTER 4. DISCUSSION

CHAPTER 4. DISCUSSION.

In this discussion the results from chapter three from the three study groups, for reasons that will be made clear, are examined as a whole.

On first examination of the RFLPs found in study group one, there appears to be one polymorphism, (Bgl I probed with the 5' Renin) that shows a significant difference between the hypertensive and control subjects (χ^2 $p = 0.0002$). The other RFLPs studied showed no apparent difference, although the numbers in each polymorphic profile group were too small for statistical analysis. At the same time as the results were being analysed, a research group in Australia published results showing no association between the Hind III / 3' Renin polymorphism and hypertension in 29 hypertensives compared with 231 control subjects. (Morris. 1988)[42]. However, after the preliminary analysis of our blood pressure study group, there was found to be a distinct ethnic difference in the RFLP profiles of white Caucasian and black Afro-Caribbean subjects and this led to a further assessment of the ethnic grouping within our hypertensives and controls. Ethnic details of the hypertensive subjects were not taken on the initial samples but from the records held at St. George's Hospital, about a third of the hypertensive clinic patients, were from Afro-Caribbean extraction. This compared with only 5% who participated in the blood donation service, at Tooting, from where the control group of subjects were obtained. The RFLP difference between hypertensives and

controls was therefore attributed to the difference in the ethnic component of the two groups and in further future studies details of ethnic origin were obtained.

The second study group, all white Caucasians, with a normal distribution of blood pressure for the age range of 25 - 47, were chosen to investigate the association of physiological measurements with polymorphic profiles. Plasma ANP and aldosterone were measured but unfortunately it was not possible to assay for plasma renin. Urinary sodium was also measured.

There was no correlation between any of the measurements, blood pressure , plasma ANP and plasma aldosterone, when tested against each other (figures 24 - 27) however there was a negative correlation between plasma aldosterone and urinary sodium. (figure 28. $r = -0.47$, $p = 0.036$.) which would be expected from the classical effect of aldosterone on the absorption of sodium in the renal tubular system.

To obtain a more accurate picture of the correlation between physiological measurements involved in blood pressure it would be necessary to take serial samples during a 24 hour period, as most biochemical hormones have fluctuating levels over this period. Also in comparing different subjects it would be necessary to control the dietary intake of sodium, potassium and volume of liquid.

The range of values for the different physiological measurements were arranged in the form of a scatter-gram for each polymorphic profile to ascertain cosegregation of blood

pressure , ANP, aldosterone and sodium with the various RFLPs. From the visual inspection of figures 29 - 32 there was no striking clustering of values for any of the RFLPs but statistical tests could not be obtained as the numbers involved for some of the profiles were too small. Linkage disequilibrium between each RFLP in the second study group were not statistically analysed because of the small numbers in some of the profiles but it was noted that for the Taq I and Bgl II RFLP for renin that they were in strong linkage disequilibrium. This might be due to a region associated with the renin gene being highly conserved for maintaining a regulatory mechanism, however it might just be a consequence of their physical proximity at the renin gene locus or that this polymorphism was of recent origin. In the larger blood pressure study group this linkage disequilibrium was lower, with nine of the one hundred and three profiles changing but it was difficult to estimate the true values as the level of crossing over occuring in the heterozygote group could not be determined.

In the third study group it was shown from the RFLPs in Caucasian and Afro-Caribbean subjects (tables 19 - 23, figures 33 - 37) that there was no association between the upper or lower quintiles of blood pressure for any of the polymorphisms, in either of the ethnic groups, combined or separate. (χ^2 test statistic from log linear model < 2). However, the model shows a clear ethnic difference for the groups as a whole, in their associations with all but one

(Hind III / 3'Renin , p = 0.26) of the five RFLPs.

Table 24. shows the relationship between the age-adjusted plasma active renin concentrations and the various allelic polymorphisms in subjects, not on treatment for hypertension, in whom renin assays were performed. The characteristics of the black Afro-Caribbean and the white Caucasian subjects representing the upper and lower quintiles for blood pressure are shown in Table 25. A number of the subjects within the upper quintile group were on antihypertensive treatment and although this had no affect on the polymorphic profiles these subjects were not used when comparing renin levels with each polymorphism. As the Afro-Caribbean group was marginally younger overall than the Caucasians, the results were age adjusted where appropriate. Plasma active renin concentrations (Figure 41.) (geometric mean [95% CL.]) were found to be significantly lower in the Afro-Caribbeans, (11.4 uU / ml. [7.8 - 16.5]) than in the Caucasians (26.8 uU / ml. [19.3 - 37.4]).(p = 0.001) supporting the finding by Meade. (1983)[43].

Figure 40. shows the relationship between the plasma active renin concentrations and the various allelic polymorphisms using the renin probe in untreated subjects on whom renin assays were performed. The ethnic influence for renin values was corrected and there was a significant difference between the renin values from one homozygote to the other for the Bgl I / 5'Renin polymorphism (p = 0.009) but not for the other polymorphisms. However this finding would have to be

further verified with larger numbers as between each profile group there was a disproportionate number of Afro-Caribbeans to Caucasians.

The glucocorticoid receptor gene probe and the kallikrein gene probe were obtained towards the end of the study and only initial studies are reported in this thesis.

There was no association with the polymorphism found using the GR gene probe on Bcl I digests with blood pressure but there was a distinct ethnic difference in allele frequency in Afro-Caribbean and Caucasian subjects. (Table 26.)

This was in contrast with preliminary evidence from families in the Ladywell Blood Pressure study, carried out by the MRC blood pressure unit, Glasgow, that found a higher frequency of the larger allele in subjects that had a family history of high blood pressure and a lower frequency in subjects that had a family history of low blood pressure, compared to an overall population group. (Davidson. 1990)[44]

The Glucocorticoid receptor is one of a family of receptors in which studies indicate that the receptor gene products are involved in regulatory roles capable of modulating gene expression leading to changes in protein synthesis within cells.(Evans.1988)[45].

Although the actual role of human kidney kallikrein is still unclear there is now evidence to suggest that it is involved in the mediation of the renin - angiotensin system. Reduction in kinin formation due to a high production of kininase II and lower formation of tissue kallikrein results in an

increased release of the vasoconstrictor angiotensin II and a reduced production of prostaglandin E, a vasodilator. (Sharma.1988)[46]

Studies in essential hypertension have shown that there is a marked decrease in the excretion of urinary kallikrein in hypertensive subjects compared to normotensive subjects and that children of hypertensive parents have lower urinary levels than children of normotensive parents, leading to the suggestion of genetic involvement. (Margolius. 1971)[47]

Further studies, using pedigree analysis, have found a dominant allele for high total urinary kallikrein excretion, that may be associated with a decreased risk of essential hypertension.(Berry.1989)[48].

A study that was published very recently has found that blood pressure cosegregates with a polymorphism in the rat tissue kallikrein gene.(Pravenec. 1991)[49]

In this thesis preliminary experiments were carried out to detect RFLPs at the kallikrein locus that could be used to track families with high and low levels of urinary kallikrein excretion and also to compare hypertensives with controls.

Hypertension is generally considered to be a 'disease' of modern Western society and this assertion is based on the difference in the prevalence of high blood pressure in this modern society and a traditional non-Western society. (Page . 1976)[50]. While the role of many physiological factors thought to cause hypertension have been researched in

cross-cultural populations (Waldron.1982)[51], little attention has been focused on the genetic contribution that may affect these factors. Populations that are indigenous to a specific environment have adapted to it either through natural selection or by cultural practices.(Boyden. 1987)[52] and because humans evolve like any other organism, survival depends upon the successful adaptation of individuals to the specific environmental stressors. The gene frequencies of 'survival' genotypes in different populations will be affected by these stressors and modify the gene pool of the population so that specific genes become fixed.

Blood pressure is inherited like any quantitative trait. (Pickering.1961)[53]. It can be thought of as the interaction of many factors leading to the maintenance of a central value. If the failure of one of these has a genetic component, the development of high or low blood pressure will aggregate in families. However this heritable factor may well be different from one population to another and therefore when studying genetic variation in association with essential hypertension this must be taken into consideration.

This diversity is overcome when using animal models. Rodent strains for genetic hypertension are now well developed, (Levenberg. 1987)[54] and as in all species blood pressure behaves as a quantitative trait showing continuous variation which could be thought of as the net result of alleles at many genetic loci exerting a positive or even a negative effect.

In rats, by selectively breeding high and low blood pressure strains, the mechanism responsible for the blood pressure difference can then be examined by looking at discrete biochemical and physiological traits that follow a Mendelian inheritance. In this way, Rapp (1989)[55], found that an RFLP at the renin gene locus determined on an F_2 hybrid population of Dahl salt sensitive (DS) and Dahl salt resistant (DR) rats, cosegregated with blood pressure. A similar finding was found by Kurtz (1990)[56] using spontaneously hypertensive rats (SHR) and normotensive Lewis rats. Conversely another recent publication, (Lindpainter.1990)[57] showed a structural alteration of the renin gene in stroke-prone spontaneously hypertensive rats (SHRSP), that on comparison of renin genotype of an F_2 hybrid population of SHRSP rats and normotensive WKY rats showed no cosegregation with blood pressure, plasma renin activity or any other physiological parameters measured including, heart rate, blood pressure induced by sodium loading and stress, and tissue water content. These differences may be attributed by strain difference.

Recent experiments using transgenic animals, where a mouse renin gene was introduced into the genome of the rat, demonstrated that the expression of this gene caused severe hypertension. (Mullins.1990)[58].

These findings in the rat suggest that sequence variation in, or close to the renin gene locus, may contribute to the pathogenesis of hypertension and therefore in this study the

same approach has been used for looking at Human hypertension. Restriction fragment length polymorphisms were used to analyse renin gene variation and no association was found between genotype and blood pressure. A similar study on a Utah population group also reported no significant association with renin RFLPs and hypertension.
(Naftilan.1989.)[59].
The only study published on the polymorphic variation of the atrial natriuretic factor gene locus, was our own, on a preliminary study in hypertensive and control subjects.
(Webb.1990)[60]
A study using transgenic mice with an introduced ANF gene demonstrated a chronic elevation of plasma ANP leading to a decrease in arterial blood pressure without inducing diuresis or natriuresis. The ability of ANP over-expression to antagonize experimental induction of hypertension may prove informative in 'negative' gene regulation in hypertension.
(Steinhelper. 1990)[61].
The ethnic variation in the renin, ANF and glucocorticoid gene locus has not been previously reported, although similar ethnic variations are found in RFLPs at other gene loci, i.e. the insulin receptor. (Elbein. 1986)[62].

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Appendix.

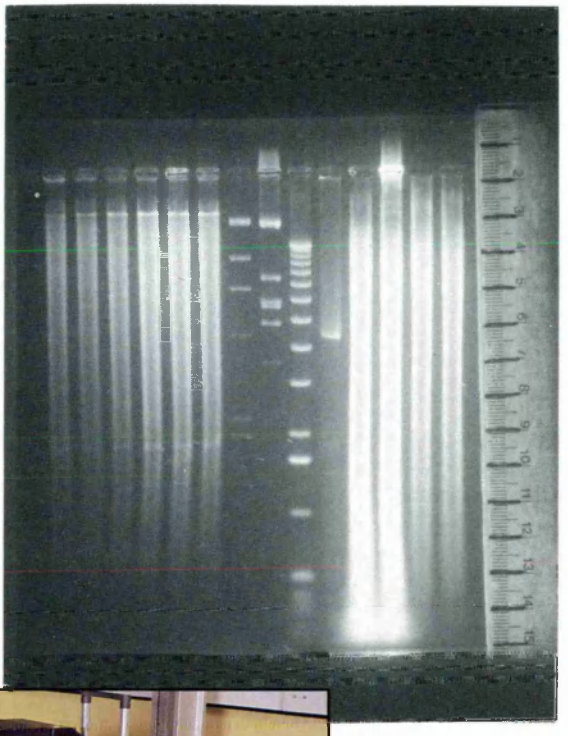
NATURE 347: 6190, 20.9.90

Polaroid at work Nº 25

DNA analysis and instant photography

AT ST GEORGES Hospital Medical School, Tooting, research into hypertension and diagnosis of genetic diseases, such as cystic fibrosis, is based on an analysis of DNA sequences - and instant photography techniques are assisting scientists in these areas of work.

Chief technician Jackie Barley of the Child Health Department is involved in research to find out whether allelic polymorphisms of candidate genes can be associated with essential hypertension. Research and medical evidence suggests that up to 60 per cent of hypertension is attributed to genetic influence. Genes with known products that effect blood pressure, renin and atrial natiuretic factor are being studied for association with hypertension. The study includes hypertensive patients having a family history of hypertension with control subjects and subjects representing the upper and lower quintiles for diastolic pressure from randomly drawn samples of Afro-Caribbean and Caucasian subjects.



Right: Digested DNA fragments, photographed on Polaroid Type 667 instant print film, using the MP-4.

Below: Jackie Barley, working with the MP-4 at St. Georges Hospital Medical School.

Part of the research is to identify and size polymorphic DNA sequences. This is assisted by Polaroid's MP-4 camera which is used to photograph digested DNA fragments separated by electrophoresis in agarose gels. These are stained with ethidium bromide and illuminated with UV light.

Barley needs Polaroid photography to give a permanent record of this part of the research. She explained: 'One of the reasons for using Polaroid photography is that you can check the quality of the restriction enzyme digest. Incomplete digestion and overloading of the sample will result in extra false bands on the autoradiograph.

'Known DNA size markers are also run on the gels and when photographed alongside a ruler provide an instant permanent record allowing DNA fragment sizes to be determined later.'

The separated DNA fragments are transferred from the electrophoresis gel and immobilised onto a nylon membrane using conventional blotting techniques (ref: E.M. Southern, *Journal of Molecular Biology* 1975, Vol 98, pages 503-517). Radioactive c-DNA gene probes are hybridised to the complimentary sequences of DNA on the membrane and visual-

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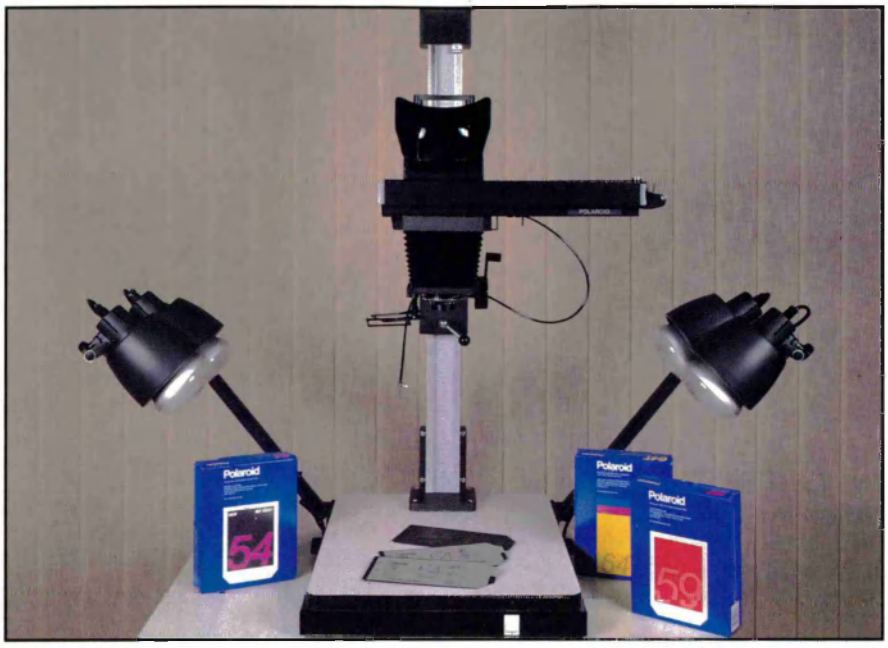
'It's reassuring to have a permanent visual record'

- Jackie Barley, St Georges Hospital Medical School.

The multi-purpose MP-4 copy camera



Above: The Polaroid MP-4 camera system and film.

POLAROID's MP-4 multi-purpose camera is a complete photographic studio in miniature, a camera and copy stand combined for making instant images on more than two dozen instant films, including 35mm, 3¼ x 4¼-inch, 5 x 4-inch and even 10 x 8-inch formats when upgraded.

The unit can be adapted to handle a wide range of applications, including specimen photography, photomicrography, photomacrography and general copy work. The camera body slides on a column over a baseboard, or it can be used, independent of its copying stand, as a conventional studio camera.

Features include eye-level reflex viewing, six interchangeable flat field lenses, self-cocking shutter, easily interchangeable film holders, infinitely-adjustable built-in lighting and a camera head that rotates through 360 degrees.

The modular design of the camera, together with its wide range of accessories, means that photographers can build a system tailor-made to specific requirements, with the option of upgrading as needs change.

Peel-apart print films

THE MP-4 takes any one of Polaroid's vast range of 3¼ x 4¼-inch pack films. According to the film in use, they offer instant images in black and white or colour.

Processing is by Polaroid's peel-apart method. Two tabs are pulled from the film back, as soon as the exposure has been made and the print emerges with the second one.

After a short wait, the print is peeled away from its backing. Development time, on average, is 30 seconds for black and white, 60 seconds for colour.

There are over fifty different films in Polaroid's range. Type 669 is just one, available in glossy or the Type 669S silk finish surface. The silk finish is one that has, since its launch, proved tremendously popular with many different types of photographer.

It is less reflective, less prone to marring by finger-prints and dries faster than its glossy counterpart, so that it can be mounted in a presentation folder or safely stacked with other prints immediately after exposure without the risk of sticking.

DNA ANALYSIS AT MEDICAL SCHOOL

◀CONTINUED FROM PREVIOUS PAGE

ised by exposing it to x-ray film to create an autoradiograph.

As Jackie Barley says: 'Polaroid photography is actually essential. There are so many procedures that can go wrong, it's reassuring to have a permanent visual record to refer to.

The Polaroid camera is also used by senior molecular geneticist Rohan Taylor in prenatal genetic disease diagnosis. A key area of this work is cystic fibrosis analysis, where DNA is analysed from chorionic villus (placenta) samples.

Rohan Taylor uses the Polaroid MP-4 to photograph agarose gels with amplified DNA fragments stained with ethidium bromide. These gels have a limited fluorescent time before the bands irrecoverably fade. Again, the photographs highlight band locations, showing differences in DNA sequence and indicating the presence of mutations in the cystic fibrosis gene. She also uses the camera for muscular dystrophy diagnosis (where the absence of an amplified DNA fragment is indicative of the disease state).

The system is now the recognised method for documentation and publication of electrophoresis gel analysis.

Reader Service Nº 930

SUPPLEMENT

Results of log-linear modelling to assess whether the association between quintile of blood pressure and polymorphism is different in each ethnic group.

I have assumed that each subject has two genes, therefore the total number in the gene frequency tables is twice that of the tables of the allelic distribution.

The notation used to describe the log-linear model is as follows; the initial letter of each factor is used, i.e. P denotes Polymorphism etc. Each term in the model is in parenthesis, i.e. [E] denotes that a term for Ethnic group is in the model. When there are two factors in parenthesis this denotes an association term in the model, i.e. [PE] denotes that the association between Polymorphism and Ethnic group has been included into the model. The simplest model that has been fitted to the data is [Q][E][P], this is the independence model (all terms in the model are independent of each other).

Chi-square tests of association have been performed on the Quintile by Polymorphism subtable for the AFC and Caucasians separately. When these two tests give different conclusions then we would expect the [PEQ] (second order interaction term) in the log-linear model to be significant. A significant second order interaction implies that there is a difference in the associations between two factors, at different levels of the third factor.

The P-values stated for each log-linear model are generated from testing the hypothesis that the model doesn't 'fit' the data. Therefore a significant P-value ($P<0.05$) indicates a significant 'lack of fit' and only models that do 'fit' the data ($P>0.05$) are considered for the '*best*' model.

Occasionally tables occurred where one column or row sum was zero. This happens when no subjects are placed in this category i.e. There are no caucasians with a TT allele for Taq1 digests. I have assumed that this phenomenon has occurred due to the probability of observing a subject in this cell being low and not that it is impossible to observe this particular combination of factors. I have therefore added a small number (0.1) to each cell in the observed table. This removes the possibility of division by zero or an extremely small number and also has the effect of making the P-values more conservative (larger).

There does appear to be some evidence that there are different associations between Polymorphism and Quintile of blood pressure for each ethnic group in the 5' Renin probe on Bgl I digests. In this case there is a significant association between these two factors in Afro-caribbeans but in Caucasians the Polymorphisms and Quintile of blood pressure appear independent.

In all the other digests there was only a significant association between Polymorphism and Ethnic group except for the Hind III digests where there were no significant associations.

5' Renin probe on Bgl I digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	Polymorphism B
AFC	Lower	25	23
AFC	Upper	16	36
Caucasian	Lower	37	17
Caucasian	Upper	37	13

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	**4.6875**	**1**	**0.0304**
Caucasian	0.3800	1	0.5376

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	24.56	4	0.0001
[Q][PE]	**5.41**	**3**	**0.1438**
[E][PQ]	22.95	3	0.0000
[P][EQ]	24.25	3	0.0000
[PE][PQ]	3.80	2	0.1497
[PE][QE]	5.10	2	0.0780
[QE][PQ]	22.63	2	0.0000
[PE][PQ][QE]	3.76	1	0.0524

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	**19.15**	**1**	**0.0000**
[PQ]	1.61	1	0.2045
[EQ]	0.31	1	0.5777
[PEQ]	3.76	1	0.0524

There is a highly significant association between Polymorphism and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][PE], i.e. the model with an association term between Polymorphism and Ethnic group. The second order interaction has a P-value of 0.0524, this is close to the 0.05 level which is usually taken to imply statistical significance. Thus it is possible to conclude that there is evidence to suggest a different association between Polymorphism and Quintile for AFC and Caucasians, but the evidence is not particularly strong.

Expected frequency table using this model

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	20.5	29.5
AFC	Upper	20.5	29.5
Caucasian	Lower	37.0	15.0
Caucasian	Upper	37.0	15.0

Allelic distribution results

Observed frequency table

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	8	9	7
AFC	Upper	4	8	14
Caucasian	Lower	12	13	2
Caucasian	Upper	13	11	1

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	3.6513	2	0.1611
Caucasian	0.4638	2	0.7930

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][A]	25.36	7	0.0007
[Q][AE]	**4.34**	**5**	**0.5011**
[E][AQ]	23.38	5	0.0003
[A][EQ]	25.20	6	0.0003
[AE][AQ]	2.36	3	0.5004
[AE][QE]	4.19	4	0.3814
[QE][AQ]	23.22	4	0.0001
[AE][AQ][QE]	2.31	2	0.3148

'*Best*' model has been **bolded**.

Statistical significance of each association

Model	G^2	d.f.	P-value
[AE]	**21.02**	**2**	**0.0000**
[AQ]	1.98	2	0.3716
[EQ]	0.16	1	0.6892
[AEQ]	2.31	2	0.3148

There is a highly significant association between Allelic distribution and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits

the observed data is [Q][AE], i.e. the model with an association term between Allelic distribution and Ethnic group. The second order interaction has a P-value of 0.3148, thus it can be concluded that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	6.0	8.5	10.5
AFC	Upper	6.0	8.5	10.5
Caucasian	Lower	12.5	12.0	1.5
Caucasian	Upper	12.5	12.0	1.5

5' Renin probe on Taq I digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	16	32
AFC	Upper	13	41
Caucasian	Lower	8	46
Caucasian	Upper	7	43

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	1.070	1	0.3008
Caucasian	0.0140	1	0.9059

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	7.67	4	0.1045
[Q][PE]	**1.57**	**3**	**0.6658**
[E][PQ]	7.10	3	0.0687
[P][EQ]	7.18	3	0.0664
[PE][PQ]	1.00	2	0.6051
[PE][QE]	1.08	2	0.5815
[QE][PQ]	6.61	2	0.0366
[PE][PQ][QE]	0.30	1	0.5852

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	**6.04**	**1**	**0.0140**
[PQ]	0.51	1	0.4751
[EQ]	0.43	1	0.5120
[PEQ]	0.30	1	0.5852

There is a significant association between Polymorphism and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][PE], i.e. the model with an association term between Polymorphism and Ethnic group. The second order interaction has a P-value of 0.5852, thus it can be concluded that there is no evidence to suggest a different association between Polymorphism and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	14.4	36.1
AFC	Upper	14.6	36.9
Caucasian	Lower	7.4	44.1
Caucasian	Upper	7.6	44.9

Allelic distribution results

Observed frequency table

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	3	10	11
AFC	Upper	1	11	15
Caucasian	Lower	0	8	19
Caucasian	Upper	0	7	18

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	1.4917	2	0.4743
Caucasian	0.0168	1	0.8969 *

* calculated from the 2x2 table excluding the zero cells.

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][A]	9.11	7	0.2448
[Q][AE]	**1.74**	**5**	**0.8844**
[E][AQ]	8.03	5	0.1543
[A][EQ]	8.87	6	0.1811
[AE][AQ]	0.66	3	0.8826
[AE][QE]	1.49	4	0.8276
[QE][AQ]	7.79	4	0.0994
[AE][AQ][QE]	0.11	2	0.9485

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[AE]	**7.37**	**2**	**0.0251**
[AQ]	1.08	2	0.5827
[EQ]	0.24	1	0.6242
[AEQ]	0.11	2	0.9485

There is a significant association between Allelic distribution and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][AE], i.e. the model with an association term between Allelic distribution and Ethnic group. The second order interaction has a P-value of 0.9485, thus it can be concluded that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	2.1	10.5	13.0
AFC	Upper	2.1	10.7	13.2
Caucasian	Lower	0.1	7.5	18.4
Caucasian	Upper	0.1	7.7	18.8

n.b. each cell frequency has 0.1 added to it.

3' Renin probe on Hind III digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	32	16
AFC	Upper	34	18
Caucasian	Lower	43	15
Caucasian	Upper	34	12

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	0.0183	1	0.8924
Caucasian	0.0007	1	0.9793

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	**2.83**	**4**	**0.5873**
[Q][PE]	1.25	3	0.7403
[E][PQ]	2.78	3	0.4266
[P][EQ]	1.59	3	0.6611
[PE][PQ]	1.21	2	0.5468
[PE][QE]	0.02	2	0.9906
[QE][PQ]	1.55	2	0.4614
[PE][PQ][QE]	0.01	1	0.9413

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	1.58	1	0.2088
[PQ]	0.05	1	0.8231
[EQ]	1.24	1	0.2655
[PEQ]	0.01	1	0.9413

There are no significant associations in the data, therefore the model which '*best*' fits the observed data is [Q][E][P], i.e. the independence model. The second order interaction has a P-value of 0.9413, thus it is possible to conclude that there is no evidence to suggest a different association between Polymorphism and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	36.4	15.5
AFC	Upper	33.7	14.4
Caucasian	Lower	37.9	16.2
Caucasian	Upper	35.0	14.9

Allelic distribution results

Observed frequency table

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	9	14	1
AFC	Upper	8	16	2
Caucasian	Lower	15	13	1
Caucasian	Upper	12	10	1

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	0.4462	2	0.8000
Caucasian	0.0328	2	0.9838

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][A]	**4.48**	**7**	**0.7227**
[Q][AE]	1.10	5	0.9540
[E][AQ]	4.06	5	0.5413
[A][EQ]	3.87	6	0.6947
[AE][AQ]	0.67	3	0.8793
[AE][QE]	0.48	4	0.9750
[QE][AQ]	3.44	4	0.4872
[AE][AQ][QE]	0.17	2	0.9180

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[AE]	3.38	2	0.1845
[AQ]	0.42	2	0.8106
[EQ]	0.61	1	0.4348
[AEQ]	0.17	2	0.9180

There are no significant associations in the data, therefore the model which '*best*' fits the observed data is [Q][E][A], i.e. the independence model. The second order interaction

has a P-value of 0.9180, thus it is possible to conclude that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	11.2	13.5	1.3
AFC	Upper			1.2
Caucasian	Lower	11.7	14.0	1.3
Caucasian	Upper	10.8	13.0	1.2

5' Renin probe on Bgl II digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	18	30
AFC	Upper	18	36
Caucasian	Lower	7	47
Caucasian	Upper	6	44

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	0.1932	1	0.6603
Caucasian	0.0220	1	0.8821

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	15.91	4	0.0031
[Q][PE]	**0.70**	**3**	**0.8726**
[E][PQ]	15.85	3	0.0012
[P][EQ]	15.43	3	0.0015
[PE][PQ]	0.64	2	0.7246
[PE][QE]	0.22	2	0.8980
[QE][PQ]	15.37	2	0.0005
[PE][PQ][QE]	0.02	1	0.8967

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	**15.21**	**1**	**0.0001**
[PQ]	0.06	1	0.8065
[EQ]	0.48	1	0.4805
[PEQ]	0.02	1	0.8967

There is a highly significant association between Polymorphism and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][PE], i.e. the model with an association term between Polymorphism and Ethnic group. The second order interaction has a P-value of 0.8967, thus it can be concluded that there is no evidence to suggest the association between Polymorphism and Quintile is different for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	17.8	32.7
AFC	Upper	18.2	33.3
Caucasian	Lower	6.4	45.1
Caucasian	Upper	6.6	45.9

Allelic distribution results

Observed frequency table

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	3	12	9
AFC	Upper	4	10	13
Caucasian	Lower	0	7	20
Caucasian	Upper	0	6	19

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	0.8785	2	0.6445
Caucasian	0.0257	1	0.8727 *

* calculated from the 2x2 table excluding the zero cells.

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][A]	16.63	7	0.0200
[Q][AE]	**1.14**	**5**	**0.9509**
[E][AQ]	16.10	5	0.0066
[A][EQ]	16.39	6	0.0118
[AE][AQ]	0.61	3	0.8944
[AE][QE]	0.90	4	0.9252
[QE][AQ]	15.86	4	0.0032
[AE][AQ][QE]	0.25	2	0.8804

'*Best*' model has been **bolded**.

Statistical significance of each association

Model	G^2	d.f.	P-value
[AE]	**15.49**	**2**	**0.0004**
[AQ]	0.53	2	0.7672
[EQ]	0.24	1	0.6242
[AEQ]	0.25	2	0.8804

There is a highly significant association between Allelic distribution and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][AE], i.e. the model with an association term between Allelic distribution and Ethnic group. The second order interaction has a P-value of 0.8804, thus it can be concluded that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using the '*best*' model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	3.6	11.0	11.0
AFC	Upper	3.6	11.2	11.2
Caucasian	Lower	0.1	6.5	19.4
Caucasian	Upper	0.1	6.7	19.8

n.b. each cell frequency has 0.1 added to it.

ANP probe on Bgl I digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	24	24
AFC	Upper	29	23
Caucasian	Lower	47	7
Caucasian	Upper	46	4

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	0.3335	1	0.5636
Caucasian	0.6761	1	0.4109

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	36.42	4	0.0000
[Q][PE]	**1.33**	**3**	**0.7213**
[E][PQ]	36.03	3	0.0000
[P][EQ]	36.10	3	0.0000
[PE][PQ]	0.95	2	0.6227
[PE][QE]	1.02	2	0.6008
[QE][PQ]	35.72	2	0.0000
[PE][PQ][QE]	0.16	1	0.6903

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	**35.09**	**1**	**0.0000**
[PQ]	0.39	1	0.5323
[EQ]	0.32	1	0.5716
[PEQ]	0.16	1	0.6903

There is a highly significant association between Polymorphism and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][PE], i.e. the model with an association term between Polymorphism and Ethnic group. The second order interaction has a P-value of 0.6903, thus it is possible to conclude that there is no evidence to suggest a different association between Polymorphism and Quintile for AFC and Caucasians,

the observed data is [Q][AE], i.e. the model with an association term between Allelic distribution and Ethnic group. The second order interaction has a P-value of 0.9794, thus it can be concluded that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	8.1	10.6	6.6
AFC	Upper	8.1	10.6	6.6
Caucasian	Lower	20.6	5.6	0.1
Caucasian	Upper	20.6	5.6	0.1

n.b. each cell frequency has 0.1 added to it.

Glucocorticoid receptor on BCl I digests.

Gene frequency results

Observed frequency table

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	15	33
AFC	Upper	8	36
Caucasian	Lower	23	25
Caucasian	Upper	20	24

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	2.0909	1	0.1482
Caucasian	0.0559	1	0.8131

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][P]	11.74	4	0.0194
[Q][PE]	**2.18**	**3**	**0.5365**
[E][PQ]	10.53	3	0.0145
[P][EQ]	11.74	3	0.0083
[PE][PQ]	0.97	2	0.6157
[PE][QE]	2.18	2	0.3367
[QE][PQ]	10.53	2	0.0052
[PE][PQ][QE]	0.90	1	0.3416

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[PE]	**9.56**	**1**	**0.0020**
[PQ]	1.21	1	0.2714
[EQ]	0.00	1	1.0000
[PEQ]	0.90	1	0.3416

There is a highly significant association between Polymorphism and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits the observed data is [Q][PE], i.e. the model with an association term between Polymorphism and Ethnic group. The second order interaction has a P-value of 0.3416, thus it can be concluded that there is no evidence to suggest a different association between Polymorphism and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Polymorphism T	B
AFC	Lower	12.0	36.0
AFC	Upper	11.0	33.0
Caucasian	Lower	22.4	25.6
Caucasian	Upper	20.6	23.4

Allelic distribution results

Observed frequency table

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	5	5	14
AFC	Upper	2	4	16
Caucasian	Lower	6	11	7
Caucasian	Upper	5	10	7

Chi-square tests of associations for each sub table.

Sub table	χ^2	d.f.	P-value
AFC	1.4459	2	0.4853
Caucasian	0.0517	2	0.9745

Log-linear models fitted to the data

Model	G^2	d.f.	P-value
[Q][E][A]	13.32	7	0.0646
[Q][AE]	**1.54**	**5**	**0.9087**
[E][AQ]	12.38	5	0.0300
[A][EQ]	13.32	6	0.0382
[AE][AQ]	0.59	3	0.8985
[AE][QE]	1.54	4	0.8199
[QE][AQ]	12.38	4	0.0148
[AE][AQ][QE]	0.52	2	0.7720

'*Best*' model has been **bolded.**

Statistical significance of each association

Model	G^2	d.f.	P-value
[AE]	**11.78**	**2**	**0.0028**
[AQ]	0.94	2	0.6250
[EQ]	0.00	1	1.0000
[AEQ]	0.52	2	0.7720

There is a highly significant association between Allelic distribution and Ethnic group, but none of the other associations are significant. Therefore the model which '*best*' fits

the observed data is [Q][AE], i.e. the model with an association term between Allelic distribution and Ethnic group. The second order interaction has a P-value of 0.7720, thus it can be concluded that there is no evidence to suggest a different association between Allelic distribution and Quintile for AFC and Caucasians.

Expected frequency table using this model

Ethnic group	Quintile	Allelic distribution TT	TB	BB
AFC	Lower	3.7	4.7	15.7
AFC	Upper	3.3	4.3	14.3
Caucasian	Lower	5.7	11.0	7.3
Caucasian	Upper	5.3	10.0	6.7